THESIS

MOLECULAR ANALYSIS OF CANCER OF THE UTERINE CERVIX

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

ZOOLOGY

BY

SAIMA WAJID

SECTION OF GENETICS
DEPARTMENT OF ZOOLOGY
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

2004

THESIS

Dedicated to
my father

THESIS

CERTIFICATE

This is to certify that **Ms. Saima Wajid** has worked on the research problem entitled **"Molecular Analysis Of Cancer Of The Uterine Cervix"** for the award of degree of **Doctor of Philosophy** under our supervision. This thesis embodies the original work of the candidate herself and has not been submitted in part or full for any degree or diploma to any other university.

05/04/2004

Co-supervisor

Dr. A. B. Mitra
Ex-Deputy Director (Senior Grade),
Institute of Cytology and Preventive Oncology
Indian Council of Medical Research,
Maulana Azad Medical College,
New Delhi. 110 002.
INDIA.

Supervisor

Dr Mohammad Hayat
Chairman,
Department of Zoology,
Aligarh Muslim University,
Aligarh. 202 002.
INDIA.

Acknowledgements

Acknowledgement

I express my sincere thanks to Dr A B Mitra Deputy Director Senior Grade and Officer-in-Charge, Institute of Cytology and Preventive Oncology (ICMR) Maulana Azad Medical College New Delhi, not only for his guidance and supervision throughout my work but also for providing me with the infrastructural facilities to carry out my work Words cannot fully express the debt that I will always owe him

This is a deep sense of my sincere thanks and respect to my supervisor Dr M Hayat Chairman, Department of Zoology Faculty of Life Sciences A M U Aligarh for his help and cooperation, and showering his blessings through many different ways during my research work

I extend my deep sense of sincere thanks to Dr Asim Azfer Department of Zoology, Faculty of Life Sciences, A M U Aligarh, for his friendly and sincere advises gave me a lot of courage and confidence throughout my research work

My heartfelt thanks to Department of Science and Technology for offering Senior Research Fellowship

My thanks are also due to Dr S Zuhoor Qasim, Former Member Planning Commission, Vice Chairman, Society for Indian Ocean studies and Dr Ishraq Ahmad Advisor, SERC, Department of Science and Technology, for their invaluable suggestions and support during this work, whenever required I am grateful and thankful to Prof Akhtar Hussain, Deptt Of Biosciences Jamia Millia Islamia for his advises, encouragement and cooperation during the tenure of my research

I am also highly grateful to Mr J K Sharma Senior Research officer, for his constant support throughout the experimental work I extend my deep sense of sincere thanks to Dr Mausumi Bharadwaj, Asstt Director Institute of Cytology and Preventive Oncology, for compilation of my thesis and papers.

Thanks to Dr Rita Ghosh, Director, Double Helix, for providing guidance and training in immunohistochemistry technique

I express my sincere thanks to Mr Mahendra Pratap, Sr Technical officer for his technical assistance and moral support throughout my experimental work

and Mr I U Khan, ex-Technical Officer, Institute of Cytology and Preventive Oncology for providing slides for microdissection and immunohistochemistry

I cannot forget to thank Dr. Anita Sharma, Sr Technical officer and Mr R Suresh, Research Officer, Institute of Cytology and Preventive Oncology, for their cooperation and any-time help. My special thanks are also due to Ms Thankamma James, Mr Raghunandan Manjhi and Mr. Shivlal for their help and cooperation in carrying out the experimental work in lab.

It is my pleasure to thank Dr. N. S. Murthy, Asstt. Director, Ms. Shashi Sharma, Senior Research officer and Ms. Sarita Sardana, Institute of Cytology and Preventive Oncology, for their help in statistical analysis of my results.

I express my sincere thanks to Dr Pushpa Sodhani, Asstt Director and Dr Sanjay Gupta, Senior Research officer, Institute of Cytology and Preventive Oncology, for their guidance in study of histopathological slides for microdissection

I also thank Dr Veena Singh for providing the samples for the study.

This acknowledgement would be incomplete without thanking my colleagues and friends Dr. Azra Shaheen, Dr. Abul Farah, Dr. Suresh Hedau. Dr. Uma Kailash, Mr. Alok Mishra, Mr. Abdul Malik, Mr. Neeraj Jain and Mr. Bhupesh Prusty for their support and help whenever needed.

Greatest of all, my sincerest thanks to my parents, who have provided me with unflinching emotional support and guidance and it is because of their blessings I have been able to undertake and complete this work. It is my papa, the ideal man in my life for whom I am at this level. I have got inspiration, advise, encouragement, love and affection from him at every step of my life.

Words fail me to express my thanks to Samar, my husband and my best friend, for his devotion, efforts, support, cooperation, care, invaluable help and suggestions during the work for my thesis.

Saima Wajid

(Saima Wajid)

CONTENTS

Abbreviations

ABBREVIATIONS

α	Alpha
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ATP	Adenosine tri phosphate
β	Beta
bp	Base pair
BME	Beta mercaptoethanol
BPB	Bromophenol blue
CPM	Counts per minute
Ci	Curie
CIA	Chloroform Iso-amyl alcohol
DAB	Diaminobenzidine
dATP	Deoxy adenosine tri phosphate
dTTP	Deoxythymidine triphosphate
dGTP	Deoxy guanidine triphosphate
dCTP	Deoxy cytidine triphosphate
DNA	Deoxy ribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EtBr	Ethidium Bromide
EB	Electrophoresis buffer
EDTA	Ethylene diamine tetra acetic acid
EtOH	Ethyl alcohol
γ	Gamma
gm.	Gram
hMLH1	Human homologue of yeast mismatch repair gene 1
hMSH2	Human homologue of yeast mismatch repair gene 2
HNPCC	Hereditary non-polyposis colorectal cancer
HPV	Human papillomavirus
HR-HPV	High-risk HPV type
Kb	Kilobases

KCl	Potassium Chloride
λ	Lambda
LCR	Long control region
LOH	Loss of Heterozygosity
LR-HPV	Low-risk HPV type
μG	Microgram
μL	Microlitre
μM	Micro molar
M	Molarity
MD	Mild dysplasia
mg	Milligram
Mg+	Magnesium ions
MgCl2	Magnesium Chloride
mRNA	Messenger RNA
MSI	Microsatellite instability
MSS	Microsatellite stable
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nanogram
nm	Nanometre
OD	Optical Density
OPD	Out patient department
ORF	Open reading frame
ϕ	Phi
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PET	Paraffin embedded tissue
PNK	Polynucleotide Kinase
Rb	Retinoblastoma gene
RE	Restriction enzyme
RNA	Ribose nucleic acid
rpm	Revolutions per minute
SCC	Squamous cell carcinoma

SDS	Sodiumdodicyl sulphate
ssDNA	Single stranded DNA
cyp	Cytochrome p450
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TEB	Tris EDTA buffer
TTB	Tris triton buffer
TEMED	CN,N,N',N'-tetramethyllethylinediamine
Tm	Melting temperature
TRIS	Tris NaCl-EDTA buffer
tRNA	Transfer RNA
URR	Upstream regulatory region
UV	Ultra-violet
XC	Xylene Cyanol

Reagents

REAGENTS

1. 1M Tris (1 liter)

Dissolve 121.14 gm. of Trizma base in 800 mL. of distilled water. Adjust the pH to the desired value by adding conc. HCl.

pH	HCl
7.4	70mL.
7.5	60mL.
8.0	42mL.

Comments:

- If 1M solution has a yellow color, discard it and obtain better quality Trizma base.
- The pH of Tris solution is temperature dependent and decreases approximately 0.03 pH unit for each 1^0C increase in temperature. E.g. a 0.05M solution has pH values of 9.5, 8.9 and 8.6 at 5^0C, 25^0C and 37^0C respectively.
- Sterilize by autoclaving.

2. 5M NaCl (1 liter)

Dissolve 292.2 gm. of NaCl in 800mL of distilled water. Adjust the volume to 1 liter and sterilize by autoclaving.

3. 0.5M EDTA [pH 8.0] (100 mL.)

Add 18.61 gm. of EDTA to 80mL. of distilled water, stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (approx. 2 gm. of NaOH pellets). Sterilize by autoclaving.

Comments:

- The disodium salt of EDTA will not go into solution until the pH of the solution is not adjusted to approx. 8.0 by the addition of NaOH.

4. 10% SDS (1 liter)

Dissolve 100 gm. of electrophoresis grade SDS in 900 mL. of distilled water. Heat to assist dissolution. Adjust the pH to 7.2 by adding a few drops of conc. HCl. Adjust the volume to 1 liter. Do not autoclave.

5. Digestion buffer (for DNA extraction from cervical scrape) (100 mL.)

5M NaCl	2mL. (100 mM)
1M Tris (pH 8.0)	1mL. (10mM)
0.5M EDTA (pH 8.0)	5mL. (25mM)
10% SDS	5mL. (0.5%)

Adjust the volume to 100mL. by adding distilled water.

6. 100X TE (pH 8.1) (1 liter)

1M Tris	121.14 gm.
0.1M EDTA	37.22 gm.

Dissolve in 800mL. autoclaved distilled water. Mix it properly and adjust pH 8.1 with conc. HCl. Make up the volume to 1 liter by autoclaved distilled water.

7. 1X TE [pH 8.1] (1 liter)

1M Tris	10mL. (10mM)
0.5M EDTA	2mL. (1mM)

Adjust the volume to 1liter by adding distilled water.

8. Lysis Buffer [for DNA isolation from biopsy] (100mL.)

3%SDS in 2X TE (pH 8.1)

100X TE	2mL.
10% SDS	30mL.

Make up the volume to 100mL. by adding distilled water.

9. Proteinase K

Stock solution is 20mg./mL. of distilled water.

Storage temperature is -20^0C.

Concentration in reaction is 100 microgram per mL.

Reaction buffer is 0.01 M Tris (pH 7.8)

0.005 M EDTA

0.5% SDS

Reaction temperature is 37-56^0C.

Comments:

Proteinase K (pK) is a highly active protease of the subtilisin type that is purified from the mold Tritirachium album Limber. The enzyme has two binding sites for Ca++, which lie some distance from the active site and are not directly involved in the catalytic mechanism. However, when Ca++ is removed from enzyme, approx. 80% of the catalytic activity is lost because of long range structural changes (Bajorath et al. 1989). Because the residual activity is usually sufficient to degrade proteins that commonly contaminate preparations of nucleic acids digestion with pK is usually carried out in the presence of EDTA (to inhibit the action of Mg++ dependent nucleases).

10. Phenol (Mol. Wt. 94.11)

- Put the bottle at 65^0C in water bath for overnight.

- To 200mL. of phenol add 0.2 gm. of 8-Hydroxyquinoline (Mol. Wt. 145.16) i.e. 0.1%
- Add 200mL. 1X TE.
- Add 200µL β- mercaptoethanol
- Incubate at 37^0C overnight.

11. 3M Sodium Acetate (Anhydrous) pH 5.2 and 7.0

Sodium acetate 246.09 gm.

Add 800mL. of distilled water and adjust the pH to 5.2 with Glacial acetic acid or the pH to 7.0 with dilute acetic acid. Adjust the volume to 1liter with distilled water and sterilize by autoclaving.

12. 1M NH_4Cl

53.5gm. of NH4Cl in 1 liter of distilled water and sterilize by autoclaving.

13. 1M $KHCO_3$

100.1 gm of KHCO3 in 1 liter of distilled water and sterilize by autoclaving.

14. Lysis Solution [for DNA isolation from blood] (100mL.)

155 mM NH_4Cl	15.5mL. of 1M NH_4Cl
10mM $KHCO_3$	1mL. of 1M $KHCO_3$
0.1mM EDTA	20µL of 0.5M EDTA

Adjust the volume to 100mL with autoclaved distilled water

15. SE solution [for DNA isolation from blood] (200mL.)

75mM Nacl	3mL of 5M NaCl
20mM EDTA	8mL of 0.5M EDTA

make up the volume to 200mL with autoclaved distilled water

16. Ethidium Bromide (10mg/mL)

Add 1gm of EtBr to 100mL of autoclaved distilled water. Stir on magnetic stirrer for several hours top ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature.

Comments:

- EtBr is powerful mutagen and is a moderately toxic. Gloves should be worn when working with solutions that contain this dye and a mask should be worn by weighing it out. After use, these solutions should be decontaminated by method (Appendix E "Molecular Cloning" *Maniatis* Volume III).
- Quantity to be used in agarose gel is 0.5μg/mL.

17. 10 X PBS (I liter)

NaCl	80g
KCl	2g
Na2HPO4	14.4g
KH_2PO4	2.4g

Dissolve the above in 800mL of distilled water. Adjust the pH to 7.4 with HCl. Make up the volume to 1 liter and sterilize by autoclaving. Store at room temperature.

18. 10 X TBE (1 liter)

Tris Base	108g
Boric Acid	55g
0.5MEDTA (pH 8.0)	40mL
dH_2O	800mL

Finally make the volume to 1 liter. Sterilize by autoclaving.

Comment:

A precipitate forms when concentrated solutions of TBE are stored for long period of time. To avoid problem, store the solution in glass bottles at room temperature and discard any batches that develop a precipitate. TBE was originally used in the working strength of 1X (1:10 dilution of the concentrated stock) for agarose gel electrophoresis. However, a working solution of 0.5X provides more than enough buffering power, and almost all agarose gel electrophoresis is now carried out with a 1:20 dilution of the concentrated stock. TBE is used at a working strength of 1X for PAGE twice the strength usually used for agarose gel electrophoresis. The buffer reservoirs of the vertical tanks used for PAGE are fairly small, and the amount of electric current passed through them is often considerable. 1X TBE is required to provide adequate buffering power.

19. 50 X TAE (1 liter)

Tris Base	242gm.
Glacial acetic acid	57.1mL
0.5M EDTA (pH 8.0)	100mL

Adjust the volume to 1 liter with distilled water and sterilize by autoclaving.

20. 0.1M DTT

Dissolve 3.09g of DTT in 20mL of 0.01 Sodium acetate (pH 5.2)

Sterilize by filtration. Store at -20^0C.

Comment:

Do not autoclave DTT or solutions containing DTT

21. TTB (DNA isolation from cervical scrape)

10mM Tris	1mL of 1M Tris (pH 8.0)
10mM $MgCl_2$	1mL of 1M MgCl2
300mM Sucrose	30mL of 1M Sucrose
0.8% of Triton X 100	800µl

Make up the volume to 100mL by adding autoclaved distilled water.

22. TEB (DNA isolation from cervical scrape)

10mM Tris	1mL of1Mtris (pH 8.0)
10mM EDTA	2mL of 0.5M EDTA
10mM NaCl	200µl of 5M NaCl

Make up the volume to 100mL by adding autoclaved distilled water.

23. BPB (Gel loading dye)

0.25% Bromophenol Blue

40% (w/v) Sucrose in water

(Store at 4^0C)

Comment:

- The gel loading dye increases the density of the sample ensuring that the DNA drops evenly into the well.
- They contain dyes that, in an electric field, move toward the anode at predictable rates.

- They add colour to the sample, thereby simplifying the loading process.
- BPB migrates through agarose gels approx. 2.2 fold faster than Xylene cyanol FF, independent of agarose concentration.
- BPB migrates through agarose gels run in 0.5XTBE at approx the same rate as linear double stranded DNA00bp in length. These relationships are not significantly affected by the concentration of agarose gel over the range of 0.5% to 1.4%. *[Courtsey Maniatis]*

24. Sequencing Mix

Acrylamide	28.5gm
Bis-acryl amide	1.5gm
Urea	210 gm
10 X TBE	50mL
Formamide	75mL

Make up the volume to 500mL y autoclaved distilled water. Store in amber colour bottle. Sterilize by filtering through 3MM Whatman. Keep at 4^0C.

25. 10% APS (10mL)

APS	1g
dH_2O	10mL

A small amount of 10% (w/v) stock solution should be prepared in deionized water and stored at 4^0C. APS decomposes slowly, and fresh solution should be prepared weekly.

26. Preparation of 6% Denaturing sequencing gel

Sequencing Mix	120mL
10% APS	700µl
TEMED	150µl

Pour gel immediately after adding TEMED.

26. Sequencing Dye (50mL)

95% Formamide	47.5mL
20mM EDTA	2mL of 0.5M EDTA
0.05% BPB	0.025gm
0.05% Xylene Cyanol	0.025gms
20mMNaOH	500µl of 2M NaOH

Store at room temperature.

27. Dilution Buffer (50mL)

0.1% SDS	0.5mL of 10% SDS
10mM EDTA	1mL of 0.5MEDTA

Make up the volume by adding autoclaved distilled water and store at room temperature.

28. 30% Acrylamide

Acrylamide	29gm
Bisacrylamide	1gm

Heat the solution to 37^0C to dissolve the chemicals. Make up the volume to 100mL by adding autoclaved distilled water and sterilize by filtering through 3MM Whatman.

29. Preparation of 10% non denaturing gel (for the analysis of CYP2D6 polymorphism)

30% Acrylamide	16.6mL
10 X TBE	5mL
10% APS	700µl
TEMED	35µl

Make up the volume to 50mL by adding autoclaved distilled water and pour solution immediately into the sealed glass plates after adding TEMED.

Reagents for immunohistochemistry

0.05 M Tris-HCl Buffer (For washing)

pH 7.4-7.6

Tris hydroxymethyl Methylamine	6.1gm
NaCl	8.7gm
HCl	3.7mL
Double distilled water	1ltr

Endogenous Peroxidase Blocking Solution

(To be prepared immediately before use)

Absolute Methanol	96mL
H2O2 (30%)	4mL

DAB Solution

(Must be prepared immediately before use)

Combine 1-2 drops (40-100L) of DAB Chromogen with each 1mL of DAB substrate.

Apply mixture to tissue section

Incubate tissue section for 5-15 minutes

Rinse tissue, counterstain, dehydrate, clear in Xylene and coverslip

10mM Citrate Buffer pH 6.0

(For Microwave antigen retrieval)

Citric acid anhydrous	2.1gm
Double distilled water	1 liter

(Adjust pH with NaOH pellets)

Poly- L-Lysine

(For coating the glass slides)

Poly-L-Lysine (Sigma)	10mL
Double distilled water	90mL

Migration Rates of Marker Dyes Through Denaturing Polyacrylamide Gels

% Polyacrylamide	Bromophenol Blue[a]	Xylene Cyanol FF[a]
5	35	130
6	26	106
8	19	76
10	12	55
20	8	28

Superscript a, denotes the numbers that are the approximate sizes of DNA (in nucleotides) with which the marker dyes will co migrate.

Migration Rates of Marker Dyes Through Non-Denaturing Polyacrylamide Gels

% Polyacrylamide	Bromophenol Blue[a]	Xylene Cyanol FF[a]
3 5	100	460
5	65	260
8	45	160
12	20	70
15	15	60
20	12	45

Superscript b, denotes the numbers that are the approximate sizes (in nucleotide pairs) of fragments of double stranded DNA with which the dye co migrates.

[Courtesy Maniatis]

Introduction

1. INTRODUCTION

Cancer is a disease involving dynamic changes in the genome. Several lines of evidence indicate that tumorigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal cells into highly malignant derivatives. Genomes of tumor cells must acquire increased mutability in order for the process of tumor progression to reach completion in several decades (Loeb, 1991). This increased mutability could be a result of malfunctioning of genomic "caretaker" systems, which are responsible for the maintenance of genomic integrity by a complex array of DNA monitoring and repair enzymes (Lengauer et al., 1998). Tumor suppressor genes fall into this category, which, in response to DNA damage, elicits either cell cycle arrest or to allow DNA repair to take place or apoptosis, if damage is excessive. The type of genetic damage might take different forms such as gene amplification, loss of heterozygosity, point mutations and viral DNA integration (Lazo et al., 1999). It is ~~eminently plausible, indeed~~ likely, that the enzymes which guard us against the systemic entry of toxic exogenous chemicals ~~should~~ may play some ~~part~~ role(s) in the etiology of human cancers caused by chemicals.

Cancer of the uterine cervix is the second most common cancer among women worldwide The estimated new cancer cervix cases per year are 5,00,000, of which 79% occur in the developing countries (IARC Scientific Publication, 1997) Precursors of cervical cancer traditionally occur at least a decade earlier than does the invasive cancer, supporting the temporal evolution of cervical cancer from its precursors (Koss et al , 1963; Richart 1973; Hellberg et al , 1993) These precursors, or precancerous lesions are known as dysplasia The term "dysplasia" which literally means bad molding was introduced in the late 1950s (Reagan et al., 1953). A spectrum of pathological lesions between normal epithelium to cancer occurs as mild, moderate, severe dysplastic and carcinoma *in-situ*, before developing uterine cervical cancer Extensive viral carcinogenesis studies the world over point to human papillomavirus as an important factor in cervical carcinogenesis (zur Hausen, 1991, 1996, Munoz et al 1992, Schiffman et al 1993, 1995, Bosch et al 1995, Franco et al 1995) However, experimental evidences suggest infection of HPV alone is not sufficient for transforming a normal cell into malignant one, indicating involvement of additional genetic alterations in the progression of cervical cancer, either independently or in conjunction with other etiological factors including HPV infection These genetic factors could be a cascade of genetic alterations involving either inactivation of tumor suppressor genes

(Mitra et al., 1994a; Mullokandov et al., 1996) or activation of oncogenes (Bishop and Weinberg, 1996; Peifer et al., 1997), or both. Recently, differential amplification as well as expression of *erbB-2* and *beta-catenin* was reported in cervical cancer (Mitra et al., 1994b; Sharma et al., 1999; Pinion et al., 1991).

Among other environmental factors tobacco smoking is an important and frequent cause of cervical cancer. The link between cervical cancer and cigarette smoke which contain carcinogenic substrates for phase 1 cytochrome P450 (CYP) and phase 2 detoxifying enzymes suggests susceptibility to this cancer may be linked to allelic variation at these loci. Individuals differ in their susceptibility to cancer, and identification of predisposing genes could allow identification of women that are at risk of cervical cancer.

In view of the above, the aim of the present study was to investigate the status of genetic alterations including loss of heterozygosity and microsatellite instability, expression of *erbB-2* and *beta catenin*, CYP2D6 polymorphism and HPV infection in cancer of the uterine cervix in order to understand the possible molecular events involved in the development of cervical cancer.

Aims and Objectives

2. AIMS AND OBJECTIVES

The aims of the present study are:

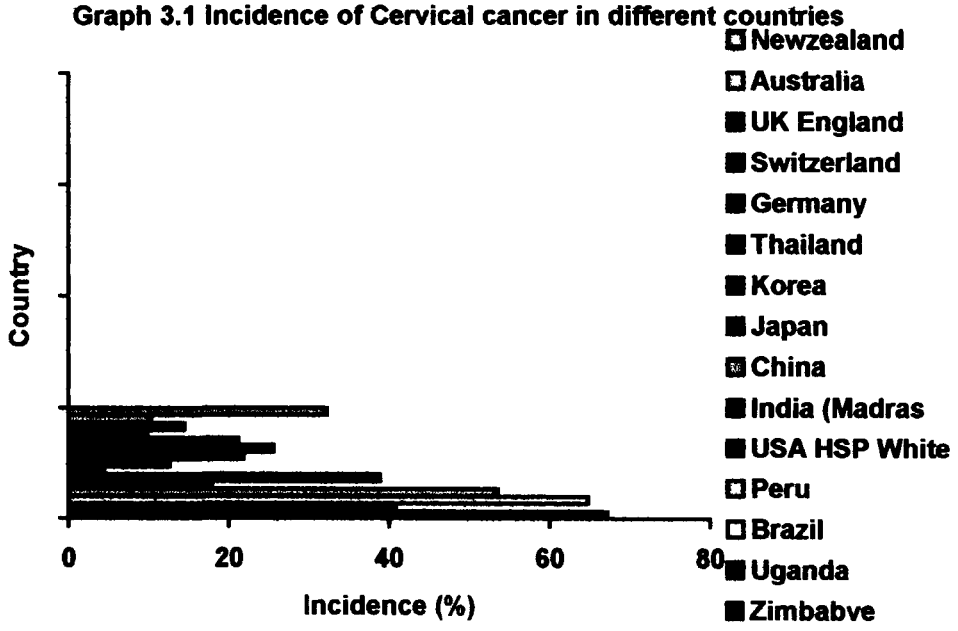
1. Analysis of microsatellite instability (MSI), using reference panel of five markers, Bat-25, BAT-26, D2S123 (AFM093xh3), D5S346 (APC) and D17S250 (Mfd215 CA) including both mono and dinucleotide repeats and their correlation with HPV infection in cancer of the uterine cervix.
2. To determine the prevalence of loss of heterozygosity (LOH) on chromosomes 3p and 5p in the cancerous lesions.
3. Detection of human papillomavirus infection (HPV) among different grades of cancerous lesions, and DNA typing of prevalent HPV types (6, 11, 16, 18)
4. Genetic polymorphism of cytochrome P450 2D6 (CYP2D6) and susceptibility to cervical cancer in the Indian population.
5. Analysis of expression of the oncogene *erbB-2/Her2neu* and *beta catenin* in squamous cell carcinoma and adenocarcinoma of cervix uteri, by immunohistochemistry.

Review of Literature

3. REVIEW OF LITERATURE

Cancer of the uterine cervix is a leading cause of morbidity and mortality among women worldwide. It is the eighth commonest cancers in the world and the second most common cause of cancer-related mortality in women worldwide. The estimated new cancer cervix cases per year are about 500,000 of which 79% occur in the developing countries (IARC Scientific Publication, 1997), with an estimated death of around 203,000 annually (Van Ranst et al., 1992). The higher rates of cervical cancer in developing countries especially in South East Asian regions are generally associated with poor hygiene and economic condition. The age adjusted incidence rate (AAR) of cancer cervix of different countries is seen in Graph3.1.

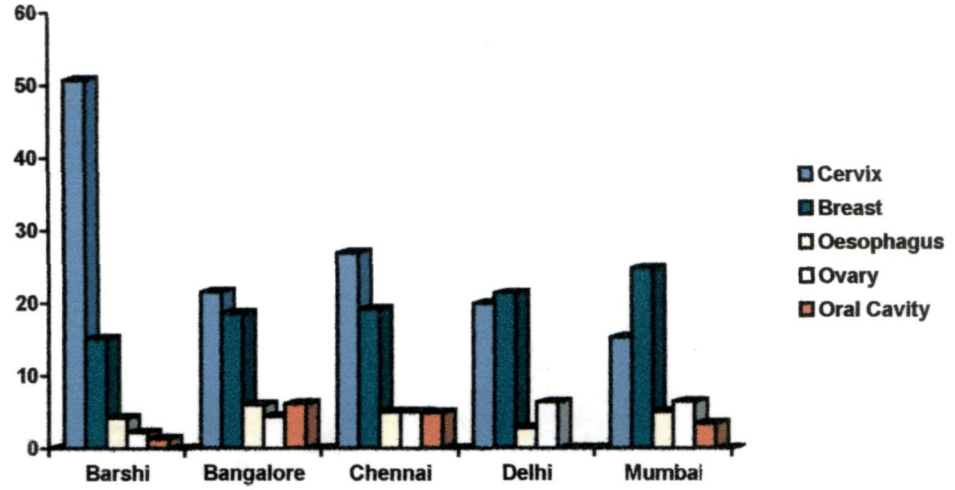
Graph 3.1 Incidence of Cervical cancer in different countries



India alone contributes about 100,000, i.e. 1/5 of this world disease burden. The truncated rate (TR) in the age group 35-64 years in Chennai, India, is even higher (99.1/100,000; 1982-95) than rate reported from Cali, Colombia (77.4/100,000, 1987-91). The cervical cancer burden in India alone is estimated as 100.000 (Shanta et al., 2000).

Cancer of the cervix is the number one cancer in females in Bangalore, Barshi, Bhopal and Chennai. While over 50% of cancers in females in Barshi were cancers of the cervix, Chennai had the highest AAR of 30.7 per 100,000. Cancers of the cervix and breast together account for over 40% of cancers in urban women and 65% of cancers in rural registry in Barshi. Incidence rate begins to rise in the early twenties in all registries and reach a peak in the 50-54 age group and only slowly thereafter. In all the registries over 90% of cases of cancer of cervix were squamous cell carcinomas. Adenocarcinoma constituted 2-4% of all cervical cancers. According to PBCR report (1990-96) cancer of the cervix was the leading site in Bangalore, Barshi, Chennai and second in Delhi and Mumbai after the cancer of breast (Graph 3.2). Most of the National Cancer Registries have been showing a declining or stationery trends since 1988. It has been estimated that every year 0.15 million new cervical cancer arise annually in India. The precancerous lesions (low grade and high grade squamous intra-epithelial lesions) account for 6 million cases prevalent in the country.

Graph 3.2 The bar chart shows the five leading sites of cancer in the five registries in females. The leading sites of cancer were decided on the basis of crude incidence rates *[PBCR consolidated report 1990-1996].*



Two sites, cervix and breast together constituted over 40% of cancer of all sites in females in urban registries and over 65% of cancers in the rural registry at Barshi (Consolidated report of PBCRs: 1990-1996).

Cervical cancer mortality trends in a number of countries have been reported by Cuzick an Boyle, (1988); Beral et al., (1994) and others. The higher rates are found in South America, Asia and Africa (Coleman et al., 1993) on the other hand, very low rates are seen in Israel and the Middle East. The rates appear to be fairly stable in these areas and this is also true for Southern and Eastern Europe. However, marked declines have been observed in both incidence and mortality of cervical cancer in North America, Western Europe and Japan. Much of the reduction has been attributed to an effect of screening (Hakama et al., 1991; Aareleid et al., 1993). However, an exception is the recent increase in rates among

young women in the United Kingdom, Australia and New Zealand (Cuzick and Boyle 1988). Even more recently, increases in cervical cancer rates among young women have been reported in Scandinavia and Eastern Europe (Beral et al., 1994). In the USA, blacks have approximately twice the cervical cancer rate than that of whites. But a recent study in Detroit of women aged 15 to 39 (Weiss et al., 1994) found that the rates were decreasing more rapidly in blacks and was nearly equal to those of whites by 1991. In United States, the incidence of mortality rates dropped to about 50% over the last 30 years due to effective cancer screening and control programs. On the other hand, cancer surveys in United Kingdom (Cook and Draper, 1984) have suggested an increase in incidence and mortality rates among young women.

Although most National Cancer Registries have been showing declining or stationary trends, however, changes in sexual behavior among adolescents, increase in oral contraceptive use, built-up of HIV epidemic, lack of infrastructure to screen, early detection and treatment of pre-cancerous lesions may reverse these trends in years to come.

Cancer of the uterine cervix shows an interestingly, highly variable and well characterized but an unpredictable developmental pattern. Cervical cancer does not arise de-novo; it involves a cascade of events and begins with the development of precancerous lesions spanning 10-15 years. These lesions may regress to normalcy, persist, and if left

untreated may culminate to invasive cancers. The precancerous lesions include a spectrum of epithelial changes, termed as cervical dysplasia, which may invade basal membranes and progress to advanced stages.

It has been shown in one of the long term prospective study that the natural history of the disease is the same as that reported in the West and every year more than 100,000 women develop this cancer in India (Luthra et al 1987) and that every alternate severe dysplasia would progress to carcinoma-in-situ, every 4^{th} moderate dysplasia and every 10^{th} mild dysplasia will show progression (Murthy, et al 1990).

RISK FACTORS

Epidemiological studies have identified a number of risk factors that contribute to the development of cervical cancer precursors and cervical cancer. These include infection with certain oncogenic types of human papillomaviruses (HPVs), and other socio-economic factors (IARC, 1995; Bosch et al., 1995; Schiffman, 1994; Walboomers et al., 1999; Franco et al., 1999; Frenczy and Franco, 2002).

Socio-economic factors

The exact cause of cervical cancer is still unknown. However, a number of predisposing factors have been identified. Major risk-factors for cervical cancer in India are considered to be socio-economic status (related to education, low income, multiple sexual partners and sexual

exposure prior to age of 18). Within the country, the highest incidence of cervical cancer is found in Chennai and Barshi, rural Maharashtra. In urban areas where the level of education is comparative high females are at reduced risk of cervical abnormalities (Graph 3.2, *Consolidated report of PBCR: 1990-96)*. Age at first intercourse, age at first child birth, high parity, number of sexual partners, promiscuity these all have been identified as possible risk factors. Progression to malignancy was found to be influenced by age at consummation of marriage (ACM). Women with consummation of marriage under 18 years of age had a 2.8-fold higher progression than those with ACM over 18 years. This may be possibly due sexual insult to the younger cervix. Earlier ACM increases the susceptibility of the cervix to the further action of carcinogenesis (Brinton and Fraument, 1986b; Luthra et al., 1987). But none of these factors attained independent significance. Sexual activity and the number of male sex partners have been identified as specific risk factors for cervical carcinogenesis. The married women of rural India are most at risk for cervical cancer. The disease has been less commonly found in the nun population and high among sex workers indicating that the partner also may play role as a possible risk factor. Studies suggest that females with history of sexually transmitted disease are more prone to cervical cancer. High parity is also associated with increased risk of developing cervical abnormalities. Regular PAP tests have dramatically reduced the risk of developing cervical cancer in developed countries.

Education and income have been accepted as major indicators of socio-economic status. The distribution of cervical cancer by educational level and income level in the hospital registry and demographic registry highlights the inverse relationship of income group and educational level

Besides other factors, females not maintaining proper hygiene after urination and intercourse are found to be at increased risk of cervical cancer. Proper washing of genitalia of both partners reduces the risk. The Indian registries show significantly lower incidence of cervical cancer in the Muslim women compared to other religious groups (Wahi et al., 1972; Jussawala et al., 1971). The religion specific incidence rate (RSI) of cancer cervix in Hindu is 24.1, Christian 21.0 and Muslim 11.2. The religious difference is strikingly marked in penile cancer incidence also. The high incidence of cancer cervix among Hindu women has been attributed to early age at coitus and poor penile hygiene on the part of male partner. The low frequency among Muslim women was attributed to the practice of ritual circumcision in Muslim men.

Human Papillomaviruses and the Development of Cervical Carcinoma

The infection of HPVs is considered to be the principal etiological agent responsible for cervical cancer (zur Hausen 1996; Das et al., 2000). More than 98% of the invasive cervical cancer shows presence of HPV. Papillomavirus viruses were first recognized many

years ago as the cause of warts on the hands and feet or condyloma accuminata on the pubic area (penis and urethra in males or vulva and vagina in females). Papillomaviruses are small non-enveloped virions with icosahedral capsid composed of 72 capsomeres (Klug and Finch 1965: Pfister, 1987; Baker et al.,1991; Pfister & Fuchs, 1994) measuring 55nm in diameter. Viruses classified in Papillomaviridae family. Cervical intraepithelial neoplasia and cervical cancer both are strongly associated with HPVs. Extensive viral carcinogenesis studies world over point to Human papillomavirus as an important etiological factor. The first IARC-sponsored case control study on HPV and cervical cancer in India carried out at Cancer Institute, Chennai documented 99% of uterine cervix were HPV positive compared to 22% in the controls. Over 90% of the cervical carcinoma has been shown to contain HPV infection (Bosch et al., 1995). Human papillomaviruses have two possible roles in malignant lesions, either due to the presence of vial early gene expression or due to the genetic damage caused by viral DNA integration (Clavel et al., 1998). After infection there is an incubation of 6-8 weeks, certain types of HPV are now officially declared to be human carcinogens (IARC 1995) representing the most important etiological agents of cervical squamous cell carcinoma and many other cancers related to squamous epithelium (zur Hausen 1996). Like any other viral infections HPV infection also remain asymptomatic or mildly symptomatic.

The circular DNA genome of all papillomaviruses can be divided into three segments of unequal size. The long control region (LCR), also called the upstream regulatory region (URR) or noncoding region, represents about 10% of the genome. The early (E) and late (L) genes are coded by about 50% and 40% of the genome respectively.

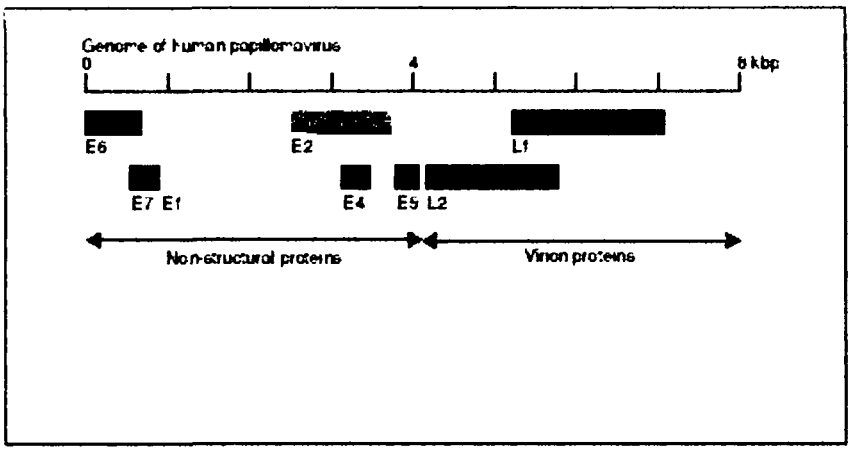


Fig.3.1 simplified organization (linearized) of human papillomavirus type 16 genome

HPV infection may be categorized into clinical and sub clinical infection. Clinical infection may be expressed in the form of condyloma accuminata. The subclinical infection is usually associated with oncogenic potential. The latent infection is usually associated with histological cervical lesion with HPV DNA. More than 70 types of HPV types have been characterized so far on the basis of differences in their DNA sequences (Favre et al., 1989a,b,c and d; Heilman et al., 1980; Danos et al., 1982; Kremsdorf et al., 1980, 1984; Gismann et al., 1977; Dürst et al., 1983., Boshart et al., 1984; Cole 1986; Beaudenon et al., 1986; Lorincz et al., 1987, 1989; Muller et al., 1987, Nuovo et al., 1988

etc.). Type specific prevalence is presented for the eighteen most common HPV types as identified by Clifford et al., 2003; Lechner et al., (1992) in meta-analysis of HPV types in invasive cervical cancers are: HPV types 6, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73 and 82 in order of descending prevalence for each subgroup analysis. Two third of invasive cervical cancer cases included in the meta-analysis were associated with HPV 16 (51.0%) or 18 (16.2%) infection. *Nubia Munoz et al., (2003) in their epidemiologic classification of* human papillomavirus types grouped fifteen HPVs as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82); three as probable high-risk types (26, 53 and 660 and 12 were classified as low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108). During the normal HPV cycle, viral DNA is maintained episomally in the nucleus of the infected cell. The detection of integrated viral DNA sequences in cervical neoplasia (Schwarz et al., 1985) is frequently associated with malignant progression, integration being more common in carcinomas than in cervical intraepithelial neoplasia (Cullen et al., 1991), with some studies reporting integration in high-grade dysplasia (Lehn et al., 1988; Fukushima et al., 1990). The persistence of both episomal and integrated copies of the HPV genome in some cervical cancers has been reported (Kristiansen et al., 1994) and most studies identify at least some carcinomas with only episomal form (Das et al., 1992).

HPV Detection

Digene Hybrid Capture Assay –II

Digene Diagnostics (Bellsville, MD) have developed HPV tests for clinical routine. This test has been recently approved in the USA, by the Food and Drug Administration (FDA). The Digene HPV test using Hybrid Capture –II technology is a signal amplified hybridization antibody capture microplate assay that utilizes chemiluminiscent detection. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA: DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA: DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for RNA: DNA hybrids and detected with a chemiluminiscent substrate, several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrids resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured. It is a simple technique that could be readily automated for large-scale use if required.

Polymerase chain reaction

PCR is currently the most sensitive method to detect human papillomavirus DNA in cervical scrapes.(Walboomer et al., 1997) It is based on *in-vitro* enzymatic DNA amplification and in principle detects

one copy of a target sequence in a given sample. To date at least 30 different HPV genotypes have been isolated from anogenital tract among which 14 are HR-HPV genotypes (16, 18, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) have been defined on the basis of their isolation from cervical carcinomas (Bosch et al.,1995; Chaouki et al., 1998). Different primers have been used for the detection of wide range of HPVs e.g. General primers –mediated (GP) PCR based amplification, selected from highly conserved region L1 and E1 of HPV genome. This assay is based on three different approaches viz.:

a). Use of degenerate primers (mixture of oligonucleotides showing nucleotide differences at several intervals)

b). Degenerate primers in addition contain inosine residues at certain ambiguous base positions,

c). Acceptance of certain degree of mismatch between primers and target DNA accomplished by reducing the stringency of primer annealing (Snijders et al., 1990) Recently a new method was introduced using GP+/GP6+ PCR based procedure which included Southern Blot hybridization of PCR products with cocktails of radioactively labeled HPV type-specific internal oligonucleotides. There are several reports on the efficacy of the two most commonly used primer sets (MY09/11 and GP5+/GP6+) used for the detection of HPV. Both primer sets amplify a wide spectrum of HPV genotypes. There was differential amplification sensitivity of HPV types between the MY-PCR and GP+-

PCR primer systems (Qu et al., 1997). It was shown that MY-PCR primer set was more robust than GP+-PCR primer set in the amplification of multiple HPV DNA types within a given sample. The GP+-PCR primers were relatively inefficient in the amplification of HPV types 53 and 61 compared to MY-PCR, whereas MY-PCR was inefficient in the amplification of HPV 35.

Detection of different HPV types

Cervical intraepithelial neoplasia and cervical cancer both are strongly associated with persistence presence of some types of human papillomavirus. Over 90% of the cervical carcinoma have been shown to contain some high-risk HPV types (Nieminen et al, 1991). The genital HPVs are classified into two groups high and low risk based on whether or not the lesions with which they are associated are at significant risk for malignant progression. The low risk HPVs such as HPV-6 and 11 are generally associated with genital warts (condyloma accuminata and flat genital warts), lesions that only rarely progress to cervical cancer. The high-risk viruses, such as HPV-16 (Favre et al, 1989; Seedorf et al, 1985), HPV18 (Cole ST et al, 1987) and HPV 33 (Beaudenon et al, 1986; Cole et al 1986), and few other are associated with CIN and cervical cancer. HPV 16, 18, 33 and 45 accounts for more than 90% of the high risk types. The MY09/MY11 primer set mediated PCR (MY-PCR) (Hildesheim et al., 1994; Manos., et al., 1989) and the

GP5+/GP6+ primer set mediated PCR (GP+-PCR) (de Roda et al., 1995) are the most frequently used amplification systems for the detection of HPV DNA in clinical samples and genotyping by type specific primers (Van Den Brule et al., 1989). There was differential amplification sensitivity of HPV types between the two systems. MY-primer set was found to be inefficient for HPV type 35 detection but was proved to be more sensitive than GP+-PCR primer set in the amplification of multiple HPV DNA types within a given sample (Qu et al., 1997). According to a recent meta-analysis (Clifford et al., 2003), it was found that HPV prevalence vary little between geographical regions. The most common high-risk HPV types in cervical squamous cell carcinoma was HPV 16 whereas, HPV 18 was predominant in adenocarcinomas and adenosquamous carcinomas followed by HPV 16. The MY09/11 primer set has been used predominantly in epidemiologic studies in North and South America and Asia (Bosch et al., 1995; Liaw et al., 1995) whereas the GP5+/GP6+ has been used primarily in Europe (de Rod Husman et al., 1995). Furthermore, various studies indicated frequent co-infections of HPV 16 and HPV6, suggesting interaction between different HPV types in multiple infections. HPV 16 and 6 appeared to have antagonistic interference in cervical carcinogenesis. Serological evidences indicate protection by HPV type 6 infection against HPV type 16 cervical carcinogenesis (Sillins et al., 1999), whereas in co-infections the E6 protein of low-risk HPV (type 6)

augments the ability of E7 of HPV type 16 or E7 of HPV type 6 coupled with E6 of type 16 increases the potency and ability to immortalize cells (Halbert et al., 1992). These studies suggest the detection of low-risk HPV types and their co-infections alongwith HR-HPVs to be important for understanding the pathogenesis of the disease and the biological behavior of HPVs during cervical carcinogenesis. Infection of multiple HPV DNA types is a risk factor for cervical intraepithelial neoplasia (Liaw et al., 1995). These observations demand for an early detection of virus types, especially those associated with cervical dysplasia and cancers, with an easy, cost-effective and simple diagnostic procedure. We. developed a new rapid and cost-effective PCR- RFLP technique, using restriction digestion with Rsa I, of the amplified products of L1 region (by MY09/11 consensus primers) (Naqvi et al., 2004). It detects five different HPV types and their co-infections simultaneously Comparing the two techniques, RFLP with restriction enzyme Rsa 1 and PCR with type specific primers, we found that RFLP using Rsa-1can detect co-infections simultaneously and more consistently than individual typing with type specific primers and was cost effective as it saves the total cost of second PCR for HPV typing with type specific primers (Aadrian et al., 1990). Use of nested PCR with degenerate primers has been described by various workers to be extremely sensitive means of detecting a wide range of HPV types (Broker et al., 2001; Harwood et al., 1999; Pizzighella et al., 1995). RFLP of the inner nested

PCR product of MY09/11 primers using *Bst E11* and a double digest of *Pst 1* and *Bgl1* (Patti et al., 2002) detected ten high-risk anogenital HPV types. Both the methods were able to detect broad range of HPV types. Most of the PCR-RFLP studies show either use of multiple restriction enzymes with two rounds of amplification. Whereas, method described in this paper, was found to be precise, with only one round amplification, followed by RFLP using single restriction enzyme, detected the five most prevalent HPV types commonly associated with cervical abnormalities and cancer. In conclusion, this method was less cumbersome, low-cost and user-friendly for the detection of HPV DNA from cervical swabs, both at clinical and research level.

CYP2D6 polymorphism in cervical cancer

Among other environmental factors tobacco smoking is an important and frequent cause of cervical cancer. Data of cancer registry reveals a positive correlation between cervical cancer and tobacco related cancers (Winkelstein, 1977). Tobacco smoke contains many potential carcinogens including polycyclic aromatics hydrocarbons, N-nitrosamines, aromatic amines, aldehydes and halomethanes. The importance of tobacco derived compounds as local carcinogens is shown by the findings that nicotine cotinine are concentrated in cervical mucus which can be mutagenic (Winkelstein, 1990; Gram et al., 1992; Burger et al., 1993), and DNA from cervical epithelial cells of smokers contains

adducts of the type expected from reaction with polycyclic aromatic hydrocarbons and aromatic amines (Simons et al., 1993). This may explain the biological plausibility of the association between smoking and cervical cancer. Cytochrome P450s constitute superfamily of enzymes crucial for the oxidative, peroxidative and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins and leukotrienes and xenobiotics including most of the therapeutic drugs and environmental pollutants (Nelson et al., 1996; Bertz and Granneman, 1997). The first report on existence of cyp enzyme or a "microsomal carbon mono oxide binding pigment", as it was called at that time, was published in 1958 by Klingenberg et al. This enzyme gave a unique 450nm optical absorption peak, and when its hemoprotein nature was recognized, it was given the name cytochrome P450 (Omura 1999). In several instances, striking differences in the activities of these enzymes among human subpopulations can be explained by allelic differences in a single gene (Nebert et al., 1990; Meyer et al., 1990). Therefore, it is likely that an understanding of these allelic differences will be important for determining an individual's risk of cancer and susceptibility to potentially toxic agents. CYP enzymes are expressed ubiquitously in different life forms: they have been found in animals, plants, fungi and bacteria (Nelson et al., 1996). They seem to be indispensable for eukaryotic species but not for prokaryotes, since some bacteria lack cyp

enzymes (Nelson, 1999). Eukaryotes need CYPs for the biosynthesis of sterols, which are constituents of plasma membrane (Omura, 1999). Eukaryotic cyp enzymes are membrane bound mostly localized to the endoplasmic reticulum, but some CYPs are also present in mitochondrial inner membranes. In order to function, cytochrome P450s require an electron transfer chain. In the endoplasmic reticulum this source is NADPH-cytochrome P450 reductase, previously called NADPH - cytochrome c reductase (Omura 1999). In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP (Gonzalez, 1990). Despite their occasionally minimal sequence similarity, all CYPs have a similar structural fold with a highly conserve core (Graham and Peterson, 1999). Most biotransformation of xenobiotics is done by enzymes from the families CYP1, CYP2 and CYP3. The CYP2 family has been under intensive study using the rat, mouse and rabbit as model system. The CYP2 family includes seven subfamilies in mammals. In the human, the most important CYPs from the viewpoint of drug metabolism are CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. The expression of CYP enzymes varies between individuals due to genetic and environmental factors and some diseases. These factors produce inter-individual variation in the rate and metabolic pathways of xenobiotics. One example of genetic factors influencing the inter-individual variation is the polymorphic expression of at least CYP2A6, CYP2C9, CYP2C19 and CYP2D6 among the

population. The frequency of poor metabolizers (PMs) varies between races and ethnic groups. Some dietary compound, cigarette smoking, alcohol and drugs may cause induction or diminution of the expression of certain CYPs (Pelkonen and Breimer, 1994; Pelkonen et al., 1998). The human genome includes only one functional gene in CYP2D subfamily, namely CYP2D6 (Nelson et al, 1996). Cigarette smoking is a risk-factor, suggesting polymorphism at loci that encode carcinogen-metabolizing enzyme such as cytochrome P450 (CYP2D6) and glutathione S transferase (GSTT1, GSTM1) may determine susceptibility to cervical cancer.

Genetic Alterations In Cervical Cancer

Although specific human papillomavirus types appear to be necessary etiological factors for most cervical cancers, HPV infection alone is not sufficient for transforming a normal cell to malignant one (zur Hausen 1994; Nishimura et al., 2000), additional genetic alterations seem to be required for their development and progression (Mullokandov, et al., 1996). These genetic changes include activation of proto-oncogenes and inactivation of tumor suppressor genes (TSGs). Knowledge of these genetic changes, in conjunction with the viral status of these cancers, might lead to improved methods of prognosis and the development of more effective therapeutic strategies. It is widely accepted that there are multistep molecular changes leading to malignant transformation from preneoplastic lesions to invasive tumors. Genetic

alterations in a tumor are important determinants for the behavior of the tumor cells. Proto-oncogenes are normal cellular genes, when inappropriately activated as oncogenes by molecular and chromosomal rearrangements in coordination with cellular transcription factors, cause dysregulation of growth and differentiation pathways and enhance the probability of neoplastic transformation. In contrast to proto-oncogenes, tumor suppressor genes are normal cellular genes that, when inappropriately inactivated or interaction of tumor suppressor gene products with viral oncoproteins, cause dysregulation of growth and differentiation pathways and may contribute to the development of cervical cancer. Non-lethal genetic damage lies at the heart of carcinogenesis. Carcinogenesis is a multi-step process at both the genetic and phenotypic level that results in tumor progression. The targets of the mutations are either the proto-oncogenes, tumor suppressor genes where both normal alleles must be damaged to develop cancer, or the genes that regulate cell death or apoptosis, which may be dominant or recessive, or the DNA repair genes.

Cancer related genes fall into two categories: tumor suppressor genes with a recessive phenotype and oncogenes that have a dominant effect.

Tumor suppressor genes:

Certain functions of putative tumor suppressor genes

- Induce terminal differentiation
- Maintain genomic stability

- Trigger senescence
- Induce programmed cell death
- Regulate cell growth

  Signal transducers of negative growth factors

  Regulators, e.g.PTPase γ, of tyrosine kinases
- Inhibit proteases
- Alter DNA methylase activity
- Modulate histocompatibility antigens
- Regulate angiogenesis
- Facilitate cell to cell communication

Major cellular oncogenes (c-onc) and their functions

Localization and Function		Oncogenes
Growth factor related	:	*sis, hst/int-2*
Receptor-related	:	*erbB-1 (EGFR), erbB-2 (HER-2/neu) erbB-3 Ros, blyn, fms, trk, kit, sea, LA-1*
Signal transducers	:	*ras, N-ras, K-ras, Ha-ras, src, fes, raf, abl, yes, fgr, lck, nck, fyn, gsp, gip, mos, fps, Grb-2, mos, sos*
Nuclear oncogenes	:	*myc, myb, fos, jun, erb A, ets, rel, ski*
Tumor suppressor	:	*Rb, p53, WT, NF-1, FAP, DCC, PRAD-1*
Regulators of cell death	:	*bcl-2*

Cytogenetic Studies

Cytogenetic analyses of tumors have revealed the association of specific chromosomal alterations with particular types of cancer, resulting in identification of the specific chromosomal regions involved in a number of cancers. The cytogenetic analyses of cervical carcinomas

have demonstrated non-random alterations of several chromosomes. A specific chromosomal abnormality associated with the condition may indicate potential candidate regions in which gene(s) affected by the predisposing lesion reside. The Knudson two-hit model of carcinogenesis (Knudson, 1971) proposed that a mutation transmitted as a dominant trait might be recessive at the cellular level and lead to cancer when the normal allele is lost or inactivated. The chromosomal *mechanisms which can lead to somatic inactivation of loci in tumors* suggest another method of determining the location of genes whose function must be lost for malignancy to develop: assay for tumor specific loss of genetic material. Studies of chromosome specific loss of heterozygosity (LOH) in tumor cells affecting a limited genomic region have supported gene localizations suggested by constitutional and tumor specific deletion or linkage analysis. LOH has been used to suggest the presence of tumor suppressor genes in a wide variety of tumors. Cytogenetic studies, although limited, have revealed non-random chromosomal changes such as i(5p), deletion at 3p, 6q and 17p (Mitra et al., 1994b; Atkin 1997), also a correlation between the common fragile sites and integration of HPV DNA (Cannizzaro et al., 1988). Mutations or deletions of a tumor suppressor gene have been shown to be frequently accompanied by loss of the remaining allele, leading to homozygous inactivation of the gene (Marshall, 1991). Several molecular studies (Mitra et al., 1994a; Mullokandov et al., 1996;

Kersemaekers et al., 1998) have identified a few frequent losses of heterozygosity (LOH) sites, suggesting possible involvement of the TSGs in the development of cervical cancer. Another common region of loss of heterozygosity is the short arm of chromosome 3 identified as 3p14 and 3p25 (Chung et al., 1992), 3p21-22 (Karlsen et al., 1994) or 3p14-or 3p14-21 and 3p22-24.1 (Yokota et al., 1989), loss of genetic material at these loci was seen in both HPV positive and HPV negative women. There was no correlation between HPV infection and 3p deletion. Loss of heterozygosity on chromosome 3 was found in both invasive cancers and cervical premalignancies, suggesting that these alterations play a role in tumor development.

Loss of Heterozygosity

Recently, with the availability of molecular cytogenetic techniques some frequent genetic abnormalities like losses of heterozygosity (LOH) consistent with the data of comparative genome hybridization (CGH), have been detected. The term 'loss of heterozygosity' was first used by Dracopoli and Fogh, (1983) to describe a reduced level of heterozygosity. Further it was noted that loss of functional gene expression was predicted by models of carcinogenesis involving the accumulation of recessive somatic mutations (Dracopoli and Fogh, 1983). Allelic loss and deletion mapping using microsatellite markers and the detection of homozygous deletions represented until

now the most powerful method to localize potential tumor suppressor genes. Frequent LOH at a given locus implies the existence of a candidate tumor suppressor gene that is important in the pathogenesis of the particular cancer under study. Currently, the consistent observation of LOH for markers at a specific location in a series of tumors of a particular histological type is considered to be a marker for loss of function of one or more tumor suppressor genes in the marked genomic region. LOH is being detected with increasing frequency in a wide variety of human tumors (Meltzer et al., 1987; Kovacs et al., 1988; Yokota et al., 1989; Vogelstein et al., 1988; Fey et al., 1989; Vogelstein et al., 1989; Lee et al., 1990; Simon et al., 1991; Wagata et al., 1991; Khosla et al., 1991).

Various tumor suppressing pathways and cancer-causing genes have been extensively studied over the past decade ((Evan et al., 2001). The clearest example of the use of LOH analysis to gain insight into tumor progression is in colorectal cancer, which offers a prototype approach to delineating the sequence of functional loss of tumor progression of malignancy. Several regions of the short arm of chromosome 3 are commonly affected in different epithelial tumors, and chromosome 3p contain several tumor suppressor genes. Frequent deletions with complicated profiles have been revealed in 3p by cytogenetic and allelotyping studies (Kok et al., 1997; Lerman et al., 2000). Frequent deletions with complicated profiles have been revealed in 3p by

cytogenetic and allelotyping studies (Kok et al., 1997; Mertens F., 1997). High frequency of LOH on chromosome 3p (47%) was reported (El-Naggar et al., 1995) in invasive HNPCC but no significant 3p loss (0.5%) in pre-invasive lesions while Mao et al., (1996) reported allelic loss (36%) for 3p in oral leukoplakia. Studies in breast cancer have revealed several regions where LOH is predictive of outcome independent of standard clinicopathological classification. LOH is believed to represent inactivation of one allele of a tumor suppressor gene by chromosomal or subchromosomal deletion (Mitra et al., 1994a). LOH studies through Southern Blotting require large quantities of intact, high molecular weight DNA and sensitivity of These LOH were detected in both SCC (68%) and AC (42%) (Larson et al., 1997) as frequent LOH at a given chromosomal locus implies the existence of a TSG that is important in the pathogenesis of the particular cancer study. LOH analysis has been done with a panel of probes for chromosomal arms 3p and 5p. LOH at 3p might be an indicator of progression since its frequency increases from 25% in FIGO stage I to 100% in stage IV cases (Larson et al., 1997). Several studies agree that within the region 3p14-22 there are two sub regions where the LOH are concentrated and frequently both are altered in the same tumor. These two regions are 3p14.2 and 3p21(Kohno et al., 1993; Chu et al., 1998). The data suggest that two TSGs are located in this chromosomal region (Huettner et al., 1998). Recently the FHIT gene has been identified within the

chromosomal region 3p14.2 which is altered in several types of tumors such as lung (Sozzi et.al.,1996), breast (Hayashi et al. 1997), esophagus (Zou et.al.,1997), cervical (Pierre et al., 1998). Another study with 75 highly polymorphic loci located on major autosome arms was used to estimate the incidence of LOH in 38 cervical cancers. LOH was found to involve 19 chromosome arms in 20-30% of the tumors. High frequency of allelic loss was found on 6p, 3p and 18q. Frequent deletions on 3p have been confirmed by many other studies. LOH at 3p were shown not only in cervical cancers but also in cervical intra-epithelial neoplasia. Mutation or deletion in a TSG is frequently accompanied by loss of the remaining allele, leading to homozygous inactivation of the gene (Marshal 1991). Chromosome deletions and loss of heterozygosity studies from a variety of neoplasms have identified the short arm of chromosome 3 (3p25 -3p14) as the location of genes involved in tumor suppression. Tumors with evidence of recurring chromosome abnormalities and high frequency allelic loss of 3p include: small cell lung cancer (SCLC)(3p22-p21) (Killary et al., 1992; Brauch et al., 1987), non small cell lung carcinoma (3p23-p21) (Kok et al., 1987), renal cell carcinoma (3p14.3-p13) (Kovacs et al., 1988), breast, ovarian and testicular cancer (3p23-p21) (Devilee et al., 1989; Trent et al., 1985; Lothe et al., 1989), squamous cell carcinoma (3p24-p12) (Gollin et al., 1994) and cervical carcinoma (Mitra et al., 1994a; Mullokandov et al., 1996). LOH studies using molecular genetic tools provided a powerful

approach to identify the minute genetic losses. In an analysis of 38 cases of cervical carcinoma, twenty one percent (8/38) of which were HPV negative, Mullokandov et al.(1996), noted that the spectrum of allelic losses observed in both HPV positive and HPV negative tumors was similar to that observed in other tumors of epithelial origin suggesting that, in general, the origin and progression of cervical cancer shares many features with other epithelial tumors. The expected exception was LOH on chromosome 17p. LOH for 17p, a presumed marker for loss of function of p53 is observed in 50-90% (Kern et al., 1989; Huang et al., 1992) of most tumors of epithelial origin but was seen in only 15% of HPV positive cervical cancer. Thus, while viral inactivation of p53 is a vital step in HPV mediated cervical carcinogenesis, other genetic changes leading malignancy in HPV positive tumors appear to be similar to those occurring in HPV negative tumors. Mitra et al. (1994a), obtained similar results. To identify the genetic events that may play a role in the development of cervical carcinoma detailed allelotype analysis was performed by Mitra et al., 1994a, LOH was observed more than 25% at sites on eleven chromosomal arms. In order to further understand the role of genetic alterations that effect in cervical carcinogenesis, status of microsatellite instability in precancerous lesions has been investigated on 5p 14-ter (Mitra et al., 1995). LOH was observed in the allelotype analysis at eleven chromosomal sites which included 1q (26%), 3p (35%), 3q (31%), 4q (46%), 5p (53%), 5q (38%),

6p (28%), 10q (28%), 11p (24%), 18p (38%) and Xq (26%) the most frequent being 4q and 5p where 5p (53%) and Xq (26%) were the two novel sites that are not reported in any other cancers (Mitra et al., 1994a) suggesting that candidate TSG may be present on these arms playing a role in the development of cervical carcinoma. Study dealing with frequent deletion sites in relation to HPV infection was also conducted. LOH on 3p revealed significant association with HPV-16/18 infection, 64% of LOH at 3p was in HPV-16/18 positive tumors. On the other hand 78.6% of LOH at 5p which was most frequent (56.3%) was in tumors without HPV-16/18 infection vs. 43.7% in tumors that had infection. In another study of 38 cases of cervical carcinoma 21% were HPV negative (Mullokandov et al., 1996) noted that the spectrum of allelic losses observed in both HPV positive and negative tumors was similar to that observed in other tumors of epithelial origin suggesting the similar origin and progression of cervical cancer as that of other epithelial tumors. LOH analysis has been extensively applied to cervical cancer leading to the identification of several chromosomal regions that are recurrently affected in this cancer. LOH analysis has been extensively applied to cervical cancer leading to the identification of several chromosomal regions that are recurrently affected in this tumor. The 3p region has been implicated in ovarian, breast, testis, lung and renal carcinoma. In cervical cancer a broad region (3p12-24) was identified as a target for LOH. The data shows that LOH occurs at an

average frequency of 48% depending on the number of marker used (Mitra et al. 1994; Mullokandov et al., 1996; Jones et al., 1992; Kohno et al., 1993; Ku et al., 1997; Karlsen et al., 1994; Huettner et al., 1998). These LOH were detected in squamous cell carcinoma and adenocarcinomas.

Table 2.1 Recurrent LOH in cervical cancer

Chromosomal Region	%age	References
3p14.1-22	48	Yokota et al, 1989, Nakamura, 1992, Mitra et al, 1994, Kersaemaeker, 1998b Mullokandov et al, 1996.
4p16	40	Hampton et al,1996, Kersaemaeker 1998b. Mullokandov etal 1996
4q21-31	32	Mitra etal 1994, Kersaemaeker etal 1998b Mullokandov et al 1996
5p13-15	17	Mitra et al 1994, Kersaemaeker1998b, Mullokandov etal 1996.
6p21.3-22	41	Mitra et al 1994, Kersaemaeker 1998b, Mullokandov etal 1996.
11p15	28	Mitra et al 1994, Kersaemaeker 1998b, Mullokandov etal 1996.
11q23	38	Hampton et al 1994, Kersaemaeker 1998b, Mullokandov etal 1996
17p13.3	24	Fujita et al 1992, Kersaemaeker 1998b, Mitra et al. 1994, Mullokandov etal 1996.
18q12.2-22	24	Mitra et al 1994, Mullokandov etal 1996. Kersaemaeker et al 1998b

[Courtesy Lazo, P.A.. The molecular genetics of cervical carcinoma. *British J. Can.* (1999)]

Microsatellite Instability

Microsatellite instability (MSI) is characterized by small deletions or expansions within these short tandem repeats in tumor DNA as compared with matching normal DNA. Repetitive sequences are common in eukaryotic genomes and vary in length of repeat unit from one to several thousand base pairs. Repeated sequences can occur in tandem array, such sequences are unique to each person. One such class of sequences in humans consists of simple tandem repeats, often a mono-di or tri-nucleotide repeat. Such repeats of 2-5 nucleotide segments are known as microsatellite DNA. Abnormalities of such repeats have been implicated in the genesis of a number of human genetic conditions.

DNA repair enzymes, "Nucleotide Excision Repair" recognize and repair large, bulky lesions in DNA. The "Mismatch Repair" pathway, in contrast, is responsible for detecting and repairing short segments of mismatched base pairs. Since microsatellite repeats fall into this category, disorders of the mismatch repair pathway lead to errors in these polymorphic segments. MSI was discovered to be the result of germline mutations in the genes that encode the components of the DNA proofreading complex. These genes are hMSH2, hMLH1, hPMS1 and hPMS2, and these are the human homologues of the bacterial *mutHLS* system. Mismatch repair may be altered by mutations in any of these genes whose product is required for functional DNA mismatch repair.

Microsatellite Instability and Development of Neoplasia

It was recently discovered that mutations in the mismatch repair genes are responsible for hereditary nonpolyposis colon cancer (HNPCC). These tumors demonstrated new microsatellite alleles in tumor DNA compared to non-neoplastic normal DNA. Greater than 80% of HNPCC tumors demonstrate microsatellite instability. (Aaltonen et al., 1994) and approximately 30% of the families with HNPCC have germline mutations in hMSH2, while another 30% mutations in hMLH1 (Lui et al.,1994; Papadopoulos. et al., 1997; Peltomaki et al., 1997). Microsatellite instability is not unique to tumors of the colon. The presence of MSI has been reported in a variety of human malignancies. (Arzimanoglou et al., 1998), including cancer of endometrium (Peltomaki et al., 1993;Risinger et al., 1993; Lim et al., 1996; Caduff et al., 1996; Kobayashi et al., 1996; Katabuchi et al., 1995; Duggan et al., 1994; Burks et al., 1994), stomach (Han et al., 1993; Aaltonen et al., 1994; Dos et al., 1996; Strickler et al., 1994; Hayden et a;.1997; Mironov et al., 1994; Keller et al., 1995; Chong et al., 1994; Semba et al 1996; Rhyu et al., 1994; Seruca et al., 1994; Wu et al., 1997; Battista et al ., 1997), bladder (Gonzalez-Zulueta et al., 1993), renal, (Uchida et al., 1994) cervical (Mitra et al, 1995; Chung et al., 2001;), ovary (Fujita et al., 1995; Sood et al., 1997; Wooster et al., 1994; Arzimanoglou et al., 1996; Phillips et al., 1996; King et al., 1995; Risinger et al., 1995; Han et al., 1993) and pancreas (Han et al., 1993; Kimura et al., 1996;

Brentnall et al.,1995), prostate (Watanabe et al., 1996; Gao et al., 1994), breast (Paulson et al., 1996; Yee et al., 1994)), cervical (Han et al., 1993), glioma (Izumoto et al., 1997), leukemia (Ohyashiki et al., 1996), testis (Peltomaki et al., 1993), esophageal (Meltzer et al., 1994)

Microsatellite instability is thought to represent a type of genomic instability, which, in turn, may predispose cells to the accumulation of other genetic alterations necessary to attain a malignant phenotype. Microsatellite is often observed in microsatellite polymorphisms because the short repeat units in these polymorphisms are susceptible to replication errors. Microsatellite instability has been reported in 35% (7/20) of invasive cervical cancers. Instability was assayed on PCR based assay by comparing the tumor DNA to that with DNA derived from normal tissue of the same patient. Although dinucleotide repeats have been used most frequently for these studies, instability has also been observed in mono, di, tri, and tetra-nucleotide repeats (Hoang et al., 1997; Zhou et al., 1997). Selection of markers as well as the number of markers used in the study, for the detection of microsatellite instability has a significant effect on the determination of MIN status. It has been recommended that for the best results it is best to utilize a combination of mono and di-nucleotide repeats. Because of differences in marker selection and total number of markers used a comparison between various studies is quite difficult. In the present study analysis of microsatellite instability was done using the Bethesda Consensus

Conference reference panel of five markers, Bat-25, BAT-26, D2S123, D5S346 and D17S250 (Boland et al., 1997).

Oncogenes in cervical cancer

Involvement of various oncogenes has been reported in several human cancers. About twenty such oncogenes were reported to be involved in cancers in human. These genes were first identified from oncogenic RNA viruses called retroviruses. These oncogenes are named after the viral strain from which they were isolated: e.g. *rel, src, myb, erb*-A, *erb*-B, *fps, yes, ros, mos, ras, abl, fes, fms and sis.* Another agent that stimulates proliferation, epidermal growth factor, like *src* increases phosphorylation of membrane proteins at tyrosines, and so it is thought that this tyrosine phosphorylation somehow controls cell proliferation. Cancer cells contain ten times more phosphotyrosine than normal cells. Amplification of cellular proto-oncogenes has been associated with tumor progression (Bishop et al.,1991), e.g. ; MYCN in neuroblastomas (Brodeur et al., 1984), small cell lung cancer (Wong et al.,1986),and various other tumor types, and *erb*B-2 (HER-2/nue) in breast (Slamon et al., 1987; Skalova et al.2003) ovarian (Slamon et al., 1989; Tyson et al., 1991; Hou et al., 1996), gastric (Houldsworth et al., 1990; Al-Kasspooles et al., 1993) and bladder carcinomas (Coombs et. al., 1991) suggesting that this gene may play a role in tumorigenesis (Bishop et al., 1991). Expression of oncogenes, including *ras, myc* and *erbB-2* has also fbeen reported in a high proportion of cholangiocarcinomas.

erbB-2 (HER-2/neu)

The oncogene *erbB-2* located on chromosome 17q has been reported to be frequently amplified and overexpressed in many adenocarcinomas and carcinoma of breast (Slamon et al., 1989), stomach (Yokota et al., 1988), renal (Freeman et al., 1989) and ovary (Slamon et al., 1989; Tyson, et al., 1991; Hou et al., 1996).

It has recently been reported that *erbB-2* oncogene is overexpressed in adenocarcinomas of uterine cervix (Kihana., et al., 1994) and in CIN and invasive cancer of the cervix (Mitra et al., 1994b; Soh et al., 2002). Earlier studies reported by Mitra et al (1994b) on frequent DNA amplification of *erbB-2* in primary squamous cell carcinoma was done by Southern Blot where chances of false negativity could be high and cell to cell analysis of amplified cells and heterogeneity of amplification in tumor was not possible. *erbB-2* amplification was studied by same authors using non-fluorescence in-situ hybridization on primary untreated squamous cell carcinoma. *erbB-2* amplification was observed in 36.6% tumors as compared to the study using Southern Blot (14%) i.e. 22% extra. However both the studies clearly indicate that *erbB-2* amplification is common in cervical cancer and is considered to be one of the mechanisms of gene over expression and experimental evidences indicate that *erbB-2* gene plays an important role in development of cancer (Zhang., et al., 1989). Gene amplification

is one of the mechanisms, besides transcriptional and translational controls or a single point mutation in the gene, which may cause an increase in tyrosine kinase activity. The *erbB-2* gene encodes a 185 kDa protein, which is a member of the epidermal growth factor receptor (EGFR) subgroup of the protein tyrosine kinase superfamily. This group of kinases is involved in the regulation of a variety of vital functions including cell proliferation, cell differentiation, and stress response. Alteration in the expression of the kinases occurs in numerous tumor types and plays an important role in tumorigenesis, cancer progression, and susceptibility to cell killing by anticancer agents. Constitutive activation of the HER-2/neu gene has been detected in sub-populations of many types of human solid tumors. In general, tumor cells, which overexpress *HER-2/nue*, are intrinsically chemoresistant to DNA damaging agents. This may be a consequence of altered cell cycle checkpoint and DNA repair mechanisms and dysregulation of apoptotic pathways. *HER-2/neu* overexpression can also enhance metastatic potential by promoting multiple steps in the invasion and metastasis process. In general, *HER-2/neu* overexpression leads to a high degree of malignancy and may predict a poor prognosis and shorter survival of the patients. Because normal critical tissues show little or undetectable expression of the gene, the *HER-2/neu* gene and its protein product are attractive targets for therapeutic approach. The *HER-2/neu* oncogene was first identified as a dominant transforming gene in chemically

induced adrenal neuroblastomas of neonate mice and was referred to as *neu*. Amplification of this gene occurs frequently in breast and ovarian cancer, and that it is associated with disease relapse and overall patient survival. Detection of overexpression can be performed by a variety of methods. Immunohistochemistry (IHC) is the most timesaving and cost-effective way. In addition, it offers advantage of *in situ* analysis. It is the identification of tissue constituents *in situ* by means of a specific antigen-antibody reaction tagged by a visible label. We followed the indirect method, which was more sensitive than the direct method. The procedure involved antigen retrieval, blocking of endogenous peroxidase, application of primary and secondary antibodies, application of peroxidase conjugated streptavidin and visualizing by diaminobenzidine (DAB) reaction. The slides were counterstained by Harris'hematoxylin.

β-Catenin

Cell-cell adhesion in tissue is mainly regulated by homotypic interaction of cadherin molecules, which are anchored to the cytoskeleton via cytoplasmic proteins, including α and β catenins. Normal epithelial cells tightly bind to each other, whereas cancer cells exhibit a looser association and invasive behavior. Cadherin, a cell-cell adhesion molecule, contains a transmembrane glycoprotein that accounts for its homotypic adhesion in the presence of calcium and

plays an important role in the organization and maintenance of tissue structure. Both β-catenin and γ-catenin binds directly to E-cadherin in a mutually exclusive pattern while α-catenin binds through its amino-terminal to β-catenin or γ-catenin, and through its carboxy terminal to the actin cytoskeleton (Hinck et al., 1994). The catenins also associate and form E-cadherin independent complexes with other cellular proteins such as the epidermal growth factor receptor (Hoschuetzky et al., 1994) the adenomatous polyposis coli (APC) gene product (Rubinfeld et al., 1993) and the proto-oncogenes *erbB-2* (Ochiai et al., 1994). In invasion and metastasis of cancers the initial step is disruption of normal cell-cell adhesion in the epithelial tissue. Coupling between adjacent cells by E-cadherin bridges results in the transmission of antigrowth and other signals via cytoplasmic contacts with β-catenin to intracellular signalling circuits that include the Lef/Tcf transcription factor (Christofori and Semb, 1999). E-cadherin function is apparently lost in a majority of epithelial cancers, by mechanisms that include mutational inactivation of E-cadherin or β-catenin genes (Christofori and Semb, 1999).

The catenins (α,β and γ) are ubiquitously expressed, cytoplasmic proteins associated with E-cadherin at cellular junctions. α-catenin can also bind P-cadherin and N-cadherin. *α-catenin* exhibits both actin binding and bundling activities. *β-catenin* also binds to N-cadherin and co-immunoprecipitates with APC. Cadherin/ catenin complexes are

linked to the cytoskeleton via a direct association between α actinin and α-catenin. Increased tyrosine phosphorylation can disrupt catenin-cadherin complexes, influencing cellular adhesion. β-catenin, a central component of the cadherin adhesion system, binds to both the cytoplasmic domain of cadherin and the amino-terminal domain of α-catenin and mediates cell adhesion. Mutated β-catenin does not link E-cadherin and α-catenin in gastric cancer cell-lines (Oyama et al., 1994). Mutation in APC or β-catenin are associated with carcinogenesis and accumulation of cytoplasmic β-catenin. The role of cytoplasmic β-catenin has been revealed to be the formation of complexes with DNA binding proteins of the T-cell factor-lymphoid enhancer factor (Tcf-Lef) family and their translocation to the nucleus, where this complex may regulate cell proliferation and inhibition of apoptosis (Korinek et al., 1997; Morin et al., 1997). Thus free cytoplasmic β-catenin behaves as an oncoprotein (Peifer et al., 1997).

β-catenin has two distinct roles in E-cadherin-mediated cell adhesion and carcinogenesis through APC gene mutation. One occurs at cell-adhesion sites, where cadherin become linked to the actin-based cytoskeleton. The other occur in the cytoplasm and nuclei and are thought to regulate cell transformation, therefore, reduced β-catenin expression at the cell membrane are associated with cancer progression, on the other hand, β-catenin in the cytoplasm binds to the transcription factor Tcf/Lef and is transferred to the nucleus where it upregulates

transcriptional activity (Korinek et al., 1997). The accumulation of cytoplasmic β-catenin is reported to result from the genetic mutation of APC and Axin (Ikeda et al., 1998) or that of β-catenin itself (Munemitsu et al., 1995) Thus overexpression of beta-catenin in the cytoplasm or nucleus is often found in cancers of various organs, including the large intestine (Takayama et al., 1998), endometrium, ovaries (Davies et al., 1998), esophagus (Yutaka Kimura et al., 1999), thyroid (Huang et al., 1998), soft tissue (Alman et al., 1997) and liver (Nhieu et al.,1999). Therefore the localization (cell membrane, nuclei or cytoplasm) of β-catenin is of considerable importance. It has been reported by Yutaka Kimura et al., (1999) that accumulation of free soluble β-catenin in the cytoplasm and nuclei frequently occurs during carcinogenesis of squamous epithelium of esophagus. β- catenin was expressed on the cell membrane in non cancerous epithelial cells while it was frequently expressed in the cytoplasm and/or nucleus in cancer cells (Takehiro, et al 2001). β-catenin is a structural component of the E-cadherin mediated cell-cell adhesion system as well as signaling molecule of the Wnt/wingless pathway (Miller, et al., 1999). In this signaling pathway (Behrens, et al., 1999; Morin, et al., 1999), β-catenin functions as an activator of T- Cell factor (Tcf)/ lymphoid enhance factor (Lef), and upregulates the transcription of downstream genes including *c-myc* (He, et al., 1998) and cyclin D1 (Tetsu, et al., 1999), which may be involved in human carcinogenesis. At present, *β-catenin* gene alterations and

abnormally accumulated *β-catenin* protein in tumor cell nuclei have been reported in variety of human malignancies, such colorectal carcinomas, hepatoblastomas, hepatocellular carcinomas, desmoid tumors, Wilm's tumor, melanomas, ovarian carcinomas, prostrate cancers (Murata et al., 2001; Koch et al., 1999; Kondo et al., 1999; Miyoshi et al., 1998; Maiti et al., 2000; Omholt et al., 2001; Ueda et al., 2001., Voeller et al., 1998). Mutations of *β-catenin* and aberrant expression of its protein have been identified in a number of different types of human malignancies. Cell-cell adhesion in tissue is mainly regulated by homotypic interaction of cadherin molecules, which are anchored to the cytoskeleton via cytoplasmic proteins, including alpha- and beta-catenin. Although Takayama et al., (1996) previously demonstrated that alpha-catenin is crucial for cadherin function in vivo; little is known about the role of beta-catenin. From an immunoprecipitation study, further Takayama et al., (1996) found that *β-catenin* forms a complex with E-cadherin not only in the normal epithelium but also in cancerous tissues. Their findings suggested that β-catenin forms a complex with E-cadherin *in vivo* and down-regulation of beta-catenin expression is associated with malignant transformation. Perturbation in the expression or function of the E-cadherin-catenin complex results in loss of intercellular adhesion, with possible consequent cell transformation and tumor progression (Huiping et al., 2001).

In view of the above review, it was noted that LOH at 3p was reported in many human cancers including cervical cancer. However, LOH at 5p was found to be a novel one and not reported in any other cancer. This study attempts to present further data on various genetic alterations and HPV infection prevalent in the cancerous lesions of uterine cervix, to have a comprehensive view.

Materials and Methods

4. MATERIALS AND METHODS

Collection of samples:

Biopsy and blood specimens

Tumor samples from 83 cervical carcinomas were collected between the year 2000 and 2003 from four major hospitals in Delhi. Clinically majority of the tissues of cancer patients belonged to stage II or III (FIGO classification) and histologically all the tumors were well to moderately differentiated squamous cell carcinoma. Corresponding blood samples were also obtained from the same patients after taking proper consent

Cervical smear for HPV testing

Cervical smears of the women who visited cancer clinics were collected in 1X PBS (Phosphate buffer saline) and transported to the laboratory for HPV testing. A detailed questionnaire with proper consent of the patient was also filled by the trained social workers, keeping in view the privacy and secrecy of the patient as per the ethical guidelines. All the samples were transported in ice at 4^0C and, stored at -20^0C tili further processing. Cervical scrapes were vortexed along with the wooden spatula, and poured in 1.5ml eppendorf tubes after removing the spatula. Then the eppendorfs were centrifuged at 2000 rpm for 2 minutes at 4^0C.

Paraffin embedded tissue sections

Paraffin embedded tissue sections were obtained from the biopsy specimens of cervical carcinoma, on Poly-L-Lysine coated slides for immunohistochemistry. The histopathology archive materials were fixed in 10% (w/v) buffered formalin to stop further tissue changes after removal from body and subsequently embedded in supporting material such as paraffin allowing sections (usually 10μ thickness) to be cut for examination by microscopy. The sections were histologically graded by experienced cytopathologists.

Preparation of Genomic DNA

DNA Extraction from tissue biopsies

1. Frozen tissue was taken out from -70^0C deep freezer and allowed to thaw.
2. Tissue is transferred to a disposable petridish, containing small amount of dry ice, and tissue was minced with the help of sterilized sharp surgical blade
3. 2 5ml of 1x TE was added to the petridish and the tissue homogenate was transferred to the falcon tube (50ml) by the help of 1ml pipette man. The petridish was rinsed with 1.5ml 1x TE.
4. Lysis buffer was added in the ratio of 1:2 i.e. 50% of the total volume i.e. 2ml in the present case.
5. Proteinase K was added to a concentration of 100μg/ml.

6. The contents of falcon were mixed and incubated at 37^0C for 1-2 hours or 50^0C for overnight.
7. Equal volume (6ml) of TE equilibrated phenol was added and mixed thoroughly on overhead shaker for 15-30 minutes.
8. The mixture was centrifuged at 3000-4000 rpm for 15 minutes at 4^0C.
9. Supernatant was transferred to a fresh falcon tube with the help of Pasteur pipette.
10. Equal volume of phenol + CIA (1:1) was added to the supernatant. (CIA = Chloroform : Isoamyl alcohol : : 24 : 1).
11. The mixture was mixed thoroughly on overhead shaker for 15-30 minutes and centrifuged at 3000-4000 rpm for 15 minutes at 4^0C.
12. Supernatant was transferred to a fresh falcon tube.
13. Equal volume of CIA was added and mixture was again mixed thoroughly on overhead shaker for 15-30 minutes and centrifuged at 3000-4000 rpm for 15 minutes at 4^0C and supernatant was transferred to a fresh falcon tube.
14. To the supernatant thus obtained 1/10 volume of chilled 3M sodium acetate solution and 2.5 volume of absolute ethanol were added.
15. The falcon was then shaken very gently and the spool of DNA was obtained.
16. The spool of DNA was transferred very carefully to an 1.5 ml eppendorf by the help of pipette man with autoclaved tip.

17. The DNA was washed in 70% ethanol twice in order to remove salts that may interfere in the amplification reactions (washing done by centrifuging for 5-10 minutes at 3000-4000 rpm).
18. 200µl of 1XTE was added to dissolve the pellet and the storage tube is left overnight at 37^0C and afterwards is stored at 4^0C.
19. The amount of DNA was measured by running 1 or 2µl aliquot in a 1% minigel with Hind III-digested Lambda DNA molecular weight marker. Using spectrophotometer did accurate measurement

DNA Extraction from blood

1. 2-5ml of heparinised blood is taken in 50ml falcon tube.
2. 20ml of lysis solution (155mM NH4Cl, 10mM KHCO3 and 0.1mM EDTA) was added to it.
3. The falcon was kept at 4^0C for one hour and then centrifuged at 4000rpm for 10 minutes at 4^0C.
4. The supernatant was discarded and the pellet was resuspended in 20ml SE (75mM NaCl, 20mM EDTA pH 8.0).
5. Proteinase K was added to a concentration of 100µg/ml.
6. 2ml of 10% SDS solution was added and the contents were mixed well in order to ensure that no cell clumps remain.
7. The falcon was incubated at 37^0C for overnight.

After this step protocol from DNA extraction from tissue biopsies was followed from step 7 to step 19.

DNA extraction from cervical smear for HPV Testing

The DNA isolation involves basically two steps first washing the pellet twice with Tris-Triton Buffer (TTB) and PK digestion in Tris-EDTA Buffer (TTB). 1.5ml of the sample containing scraped cervical cells was taken in 1.5ml Eppendorf tube and centrifuged at 5000 rpm for 5'. The pellet was again washed with chilled 1XPBS and cold centrifugation was done for 2 min at 2000 rpm. The pellet was washed twice in 1ml chilled TTB containing 10mM Tris-HCl (pH 8.0), 10mM MgCl2, 300mM Sucrose and 0.8% Triton-X 100. The pellet was collected and rewashed in 0.5ml Cold TE Buffer containing 10MM Tris-HCl (pH 8.0), 10mM EDTA and 10mM NaCl. Finally the pellet was resuspended in 200µl TE buffer supplemented with 1.25mg/ml PK and incubated at 65 degrees Celsius for 2.5hrs. The tubes were vigorously shaken every half-hour in order to allow uniform lysis of the pellet. When the solution became transparent it was assumed that extraction was completed was boiled at 95 degrees for 10 minutes for PK inactivation. Now the samples were ready for phenol chloroform extraction (if required).

Analysis of microsatellite instability

Microsatellite instability was analyzed on 83 cervical carcinomas using the Bethesda Consensus Conference reference panel of five markers, Bat-25, BAT-26, D2S123 (AFM093xh3), D5S346 (APC) and D17S250 (Mfd215 CA) (Boland et al., 1998).

Table: 4.1 Characteristics of the markers used for microsatellite instability analysis.

Name	Chr	Repeat	Primer seq	Tm(^{0}C)	Size(bp)
BAT 25	4q	TTTT.T.TTTT$(T)_7A(T)_{25}$	TCG CCT CCA AGA ATG TAA GT TCT GCA TTT TAA CTA TGG CTC	58	~90
BAT 26	2p	$(T)_5$.....$(A)_{26}$	TGA CTA CTT TTG ACT TCA GCC AAC CAT TCA ACA TTT TTA ACC	58	~80-100
APC	5q21/22	(CA)26	ACT CAC TCT AGT GAT AAA TCG` AGC AGA TAA GAC AGT ATT ACT AGTT	55	96-122
Mfd 15CA	17q11.2-12	$(TA)_7$....$(CA_{)24}$	GGA AGA ATC AAA TAG ACA AT GCT GGC CAT ATA TAT ATT TAA ACC	52	~150
AFM093xh	32p16	$(CA)_{13}TA(CA)_{15}(T/G\ A)_7$	AAA CAG GAT GCC GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	60	197-227

One primer (usually the forward) was endlabelled with [γ-^{32}P] ATP with a specific activity of greater than 500Ci/mmol (185 TBq/mmol), using Polynucleotide Kinase (PNK) and (0.1M) ditrio-threitol DTT at 37^{0}C for one hour.

[γ-^{32}P] ATP (LCP 101)
(Board of Radiation and Isotope Technology, JONAKI Laboratory)

Adenosine triphosphate labelled in the γ-position prepared by enzymatic phosphorylation of the corresponding nucleoside 5' diphosphates with very high specific activity ^{32}P orthophosphoric acid. The triphosphates are purified by HPLC using strong anion exchange column.

End labelling Mix

Primer (F)	2μL
T4 PNK	4μL
T4 PNK buffer	1μL
DTT (0.1M)	1μL
^{32}P[γATP]	3μL

Two reactions are set at 37^{0}C for one hour in thermal cycler.

Polymerase chain reaction

After endlabelling the primer is added to the PCR mix and a standard polymerase chain reaction was carried out in a 25μL reaction volume containing 30-50ng DNA, 20pmol of primers. Amplification reactions were carried out for 35 cycles at annealing temperatures ranging from 52^0-56^{0}C using 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200μM dNTPs (*New England Biolab*). The amplification was first checked on 2% agarose and finally resolved on 6% denaturing Polyacrylamide/ Urea gel (40cm x 20cm x 0.5mm) i.e. slightly thicker than the usual sequencing gel. Gel was polymerized in the presence of urea and formamide that suppresses base

pairing of nucleic acids, alkali was not used as denaturing agent because it deaminates acrylamide. The DNA was denatured by heating the PCR products at 95^0C and immediately placed in ice to prevent base pairing. Denatured DNA migrates through these gels at a rate that is completely independent of its base composition and sequence. In the presence of free radicals usually supplied by APS and stabilized by TEMED, a chain reaction is initiated in which monomers of acryl amide are polymerized into long chains whereas N,N' methylenebisacrylamide is included in the polymerization reaction, the chain becomes cross linked to form a gel.

Preparation of glass plates

- Plates were washed properly
- Cleaned the plates first with distilled water then with alcohol
- Applied siliconizing solution (Sigma Cote) on one of the plates
- Sealed the glass plates with tapes placing appropriate spacers (0.5mm)
- Placed the tabs on the two upper edges with clamps

Preparation of the Sequencing mix

- 100mL of the Acryl amide stock was taken
- To it was added 700μL of 10% APS. Mixed well, taking care of the aeration of the sample. APS provides the free radicals that drive

polymerization of acryl amide and bisacrylamide. APS decomposes slowly, and fresh solutions should be prepared weekly.

- 40μL of TEMED (N,N,N', N'– tetramethylethylenediamine) was added immediately before pouring, mixed the solution by swirling. TEMED accelerates the polymerization of acrylamide and bisacrylamide by catalizing the formation of free radicals from ammonium persulphate.
- Gel was poured keeping the plates at an angle of 45^0 slowly, from the middle taking that care no air bubble is trapped inside
- The comb was inserted and allowed the gel to polymerize for one hour at room temperature; additional acryl amide was added if the gel retracts significantly. When the gel was completely polymerized, a schieren pattern becomes visible just beneath the teeth of the comb.
- After the polymerization was complete the comb and the tapes were removed carefully and the wells were rinsed with water immediately and the small amounts of acryl amide trapped in the wells are flushed out with 1X TBE which if not cleansed create distorted bands which are difficult to interpret.
- Plates were fixed on sequencing gel apparatus
- Poured 0.5 X TBE in both the chambers of sequencing apparatus

Pre Heating of the gel

- Gel was pre heated for about 10 minutes with loading dye in 2-3 lanes prior to loading of the samples.

Conditions for running the gel

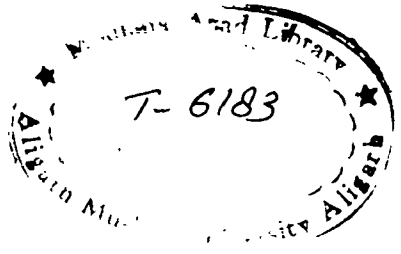
2000V, 170W, 90mA

Preparation of samples

- The samples were diluted with 50μL dilution buffer
- The samples are then mixed with 6X buffers (loading dyes), they increase the density of the sample ensuring that the DNA was dropped evenly into the well and they add color which make tracking easier and they contain dyes that move towards anode at predictable rates.
- Denatured the samples at 95^0C for 5 minutes and place immediately in ice.

Loading of samples

- The wells were gently cleaned by flushing out air bubbles from each well with the help of dropper
- Samples were loaded (2-3μL/well) immediately after taking out from ice; total time in loading should not exceed 20 minutes.
- Electrophoresis was carried out by connecting the electrodes to the power pack.



- The gel was run (for 2.5 hrs) until the marker dyes have migrated to the desired distance. Electrophoresis buffer was discarded from the reservoirs after disconnecting the power supply and glass plates were detached.

Removal of the glass plates

- After disconnecting the power supply the buffer was drained carefully as it contained radioactivity.
- The plates were rested on working table and allowed to cool (RT)
- Placing a spatula in between lifted the smaller plate spacers were also separated.
- A 3MM What man paper was placed over the gel, pressed gently and uniformly all over and lifted up along with the gel carefully taking care of the sequence of the wells by cutting a small corner at the site of the first well
- Cellophane or saran wrap was wrapped over the gel, avoiding bubbles or folds in the Saran wrap.
- The gel was placed on the gel drier (BioRad)
- Dried the gel for one hour at 80^0C

Autoradiography was done by exposing the gel on X-ray film (*Kodak*) for 24 to 48 hours.

Autoradiography

- The dried gel was placed in the cassette
- Film (Kodak) was placed on to the gel in dark room making a mark (fold or cut) at the corner to indicate the start of loading
- The cassette along with the film was kept in -20^0C for exposure
- After 24-48 hours of exposure the film was developed and fixed in dark
- The film was ready for analysis

Autoradiography produces permanent images on photographic film. The two isotopes most commonly used for autoradiography are ^{35}S and ^{32}P, both of which emit β-particles. The energies of these particles are very different. ^{35}S emits a particle with a maximum energy of 0.167MeV that can penetrate film emulsion only to depth of 0.25mm (although sufficient for the interaction between the emitted β-particle and the silver halide crystals in the film emulsions). ^{32}P emits a particle with comparatively high energy (1.71 MeV) that can pass completely through an X-ray film. To increase the efficiency, an intensifying screen is applied below the film in the cassette and exposed at low temperatures (recommended = -70^0C).

Increase and decrease in the size of repeats as compared to their normal counterpart was considered evidence of instability. Two common band patterns were observed, either a novel discrete band in tumor DNA not observed in the corresponding normal DNA or as a marked alteration of

repeat length, often heterogeneous in nature and appeared as a ladder. RMA for individual primers were also calculated. [**RMA** = Number of cases with microsatellite instability/ total number of cases].

Analysis of loss of heterozygosity

In addition to microsatellite instability, a detailed analysis of loss of heterozygosity was performed on the 83 cervical carcinomas cases with a panel of five different polymorphic markers of chromosomes 3p (3p 14.2 to 5p14.12) and 5p (5p15.1-15.3).

Table: 4.2 Characteristics of the markers used for LOH analysis.

MARKERS	AMPLIMER	AMPLICON SIZE
D3S1234	5'-CCT GTG AGA CAA AGC AAG AC-3' 5'-GAC ATT AGG CAC AGG GCT AA-3'	111bp
D3S1300	5'-AGC TCA CAT TCT AGT CAG CCT-3' 5'-GCC AAT TCC CCA GAT G-3'	236bp
D3S1313	5'-CCC CTT GGA AAA TCA CTG-3' 5'-CCA TGA ATA AGC CTT GCC-3'	233bp
D5S208	5'-ACC TGA GTC TTC ATC AAT AC –3' 5'-TCC AGA ATC ATC CAT GTT GT-3'	186bp
D5S406	5'-CCT GCC AAT ACT TCA AGA AA-3' 5'-GGG ATG CTA ACT GCT GAC TA-3'	185bp

Tumor DNA was extracted from cervical biopsy and corresponding normal DNA (constitutional DNA) was extracted from blood. End labeling of one of the two (usually forward) primer was done

with [γ-^{32}P] ATP using T4 Polynucleotide Kinase (PNK) and (0.1M) ditrio-threitol DTT at 37^0C for one hour as described earlier. Amplification was done in the thermal cycler (*Ependdorf/ Perkin Elmer*) using 1 Unit of Taq DNA Polymerase with 95^0 of initial denaturation, 52^0-56^0C annealing and a final extension of 72^0C. The amplified products were resolved on 6% Urea Polyacrylamide (denaturing) gel at 2000 Volts, 170 W and 90mA for 2.5 hrs. Steps involving preparation of glass plates, sequencing mix, preparation and loading of samples and autoradiography were similar to that described in microsatellite instability analysis.

Absence or decrease in signal intensity by more than 50% of one allele in tumor DNA compared with constitutional allele was considered evidence for LOH. The cases, which were homozygous for the allele, were taken as non-informative and were not included in the study.

HPV Detection

Detection of HPV was done by two methods:

a) Polymerase chain reaction using consensus primers

b) Digene Hybrid Capture Assay – II (DHCA-II)

HPV Detection by PCR

PCR is an *in-vitro* method for primer directed enzymatic amplification of specific target DNA sequences over a million times in a

few hours (Saiki et al 1985, Mullis et al 1987, Morris et al 1988). The detection level of PCR for HPV DNA has been estimated to be 10-100 HPV DNA molecules among 5x 10^4 cells. Several studies have been performed to assess the sensitivity of PCR to detect HPV in clinical samples. Dallas et al 1989 estimated the sensitivity of PCR to be 10 copies of virus (0.08fg of DNA) in a 10,000-cell sample subjected to 36 cycles with Taq Polymerase. Similar sensitivities have been reported by a number of subsequent studies. To exclude false positive results appropriate negative controls are mandatory. False negativity due to factors inhibiting polymerization can be avoided by proper DNA extraction.

β-globin primers PC04 (5'-CAACTTCATCCACGTTCACC-3')and GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') (Saiki et al., 1988), which produces a 268bp PCR product, were used as the internal amplification control. Extra precautions were taken to avoid cross contamination of the amplified products. The samples were screened for the presence of HPV, with positive and negative controls, by amplifying them with consensus primers (MY09-CGTCCMARRGGAWACTGATC and MY11-GCMCAGGGWCATAAYAATGG) (Manos et al., 1989) from the L1 region of HPV genome, which detects a broad spectrum of HPV types.

[M= A/C; W=A/T; Y=C/T; R=A/G]

1µl of the DNA was amplified in Thermal Cycler (*Eppendorf*), with MY09 and MY 11 consensus primers (Microsynth) of L1 region of HPV genome, covering both low and high-risk HPV types. The amplification was done using Taq DNA Polymerase 10X Buffer with 2.5mM $MgCl_2$, and dNTPs (Bangalore Genci), at initial denaturation of 95^0 for 5 minutes following 35 cycles of;

94^0 30 sec

56^0 30 sec

72^0 30 sec each

and a final extension of 72^0 for 10 minutes

8µL of the samples were loaded for resolution on 2% agarose and visualized after ethidium bromide staining.

HPV Detection Using DHCA-II

The HC-II test is a signal amplified hybridization antibody capture microplate assay for the chemiluminiscent detection of human papillomaviruses Types 6, 11, 16, 18, 31, 33, 35, 42, 43, 44, 45, 51, 52, 56, 58, 59 and 68. We used Digene Hybrid Capture Assay for the detection of High Risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 55, 56, 58, 59, 66 and 68) using single probe method. Specimens containing target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA: DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA: DNA hybrids. Immobilized hybrid cells are then reacted with alkaline phosphatase

conjugated antibodies specific for the RNA: DNA hybrids and detected with a chemiluminiscent substrate.

Reagent Preparation

All reagents supplied with the DHCA-II kit (Cat #: 5101-1096IVT) were ready to use except denaturation reagent, HPV probes and Wash buffer.

Denaturation reagent

5 drops of indicator dye (provided) was thoroughly mixed with denaturation reagent.

HPV Probe A Cocktail

For 96 wells we pipetted 140µL of HPV Probe A into 3.5mL Probe dilutent.

Wash Buffer

10mL of Wash Buffer concentrate was added to 2.9mL of distilled or deionized water.

Steps:

Deanaturation

- The hybridization tubes were labeled and denaturation reagent was pipetted out (half the volume of the specimen) and added to Control, Calibrators and Specimens and the colour of the tubes become purple.
- The tubes were incubated at 65^0C for 45 minutes. In the mean time HPV Probe mix was prepared.

Hybridization

- Pipetted 25μL of Probe A into tubes labeled "A", and to each tube was added the denatured specimen (75μL), the microtubes were covered by a plate sealer and after shaking on Rotary shaker at 1100 rpm for 3' the colour of the tubes became yellow then the tubes were incubated at 65^0C for 5 minutes.

Capture

- The contents from the microtubes were transferred to he corresponding well in Capture Microplate, sealed with a plate sealer and again shook at 1100 rpm at 20-25^0C for 65'. In the meantime wash buffer was prepared.
- Decant and blot Capture Microplate.

Conjugation

- 75μL of Detection Reagent 1 was added into each well of Capture Microplate covered with parafilm and incubated at 20-25^0C for 30 minutes.
- Decant and blot Capture Microplate.

Washing

- The plate was washed three times, raining 5 minutes on absorbent paper each time and blotted.

General Signal

- 75μL of Detection Reagent 2 was added to each well of Capture Microplate and incubated at 20-25^0C for 15 minutes.

- The Capture Microplate was read on Luminometer.

Detection of HPV types

All the positive cases were amplified individually with type specific primers for each type viz. 6, 11, 16, and 18.

Table 4.3 : Markers for detection of HPV types.

HPV Type	Primer Sequence	Amplicon Size (bp)
Type 6 (F)	5'-TAGTGGGCCTATGGCTCGTC-3'	280
(R)	5'-TCCATTAGCCTCCACGGGTG-3'	
Type 11 (F)	5'-GGAATACATGCGCCATGTGG-3'	360
(R)	5'-CGAGCAGACGTCCGTCCTCG-3'	
Type 16 (F)	5'-AAGGCCAACTAAATGTCAC-3'	217
(R)	5'-CTGCTTTTATACTAACCGG-3'	
Type 18 (F)	5'-ACCTTAATGAAAAACCACGA-3'	100
(R)	5'-CGTCGTTGGAGTCGTTCCTG-3'	

Each sample was amplified with every primer to detect the above five types using following cycles: Initial denaturation 95^0C for 5 minutes followed by 35 cycles of 94^0C for 30 sec, 56^0C for 30 sec and 72^0C for 30 sec each and a final extension of 72^0C for 7 minutes. The samples were then resolved on 3% agarose (2% agarose + 1% Nuisive) along with ϕX174 (Hae III) digested marker and stained with EtBr. The gel was visualized under UV.

Detection of HPV Types Using RFLP of L1 Amplicon

We developed a new rapid and cost-effective PCR- RFLP technique, using restriction digestion with Rsa 1, of the amplified products of L1 region (by MY09/11 consensus primers) (Naqvi et al., 2004). Detection of human papillomavirus (HPV) types 6, 11, 16, 18 and 33 including co-infections among females attending gynecological out patient department and cancer clinics, was done by restriction fragment length polymorphism (using Rsa-1), of approximately 450bp amplicon, obtained by the amplification of the L1 region of HPV genome with consensus primers MY09 /11(Manos., et al., 1989). The degenerate primers MY09/11 used for amplifying L1 region, accepts certain degree of mismatch between primers and target DNA, accomplished by reducing the stringency of primer annealing, there being always a difference of few bases in the amplicon for different HPV types, which are not distinguishable in lower concentration of agarose gels. The product (amplicon) sizes amplified by MY09/11 primers for HPV type 6 was 447bp; HPV11-449bp; HPV16-452bp, HPV18-455bp and for HPV33 was 449bp, which after digestion with Rsa 1 (recognition sequence GT'AC) gives the following band sizes

Table 4.4 showing the number of possible cuts and the sizes of the fragments resolved after the digestion of L1 amplicon with Rsa 1 for different HPV types. The bold values indicate the distinguishing bands.

Type	No. of cuts	Fragment sizes
HPV6	3	72, 67, **149** and **159**
HPV 11	3	72, **216**, 26 and **135**
HPV 16	2	72, 70 and **310**
HPV 18	4	72, 38, 85, **125** and **135**
HPV 33	3	72, 39, **236** and **102**

RFLP of the PCR products of all the HPV positive samples was done using 1 Unit of Rsa 1 (New England Biolab; Catalog No. 167 L) with NEB reaction buffer, and incubating at 37^0C for 1 hour. After digestion the products were resolved on 8% Polyacrylamide non denaturing gel with TBE buffer, after 30 minutes of staining with ethidium bromide, the products were visualized under UV/gel documentation system (BioRad) using the software Quantity One. The bands show the types of HPV present including their co-infections.

Analysis of mutant CYP2D6 alleles by Polymerase Chain Reaction

40-60ng of DNA was taken as template and standard polymerase chain reaction was carried out in a 25μL reaction volume, with 20pmol of primers. An initial strand separation step was carried out at 94^0C (4 min), and 35 cycles of: denaturation at 94^0C for 30 sec, primer annealing at 60^0C for 1 min. and polymerization at 72^0C for 1 minute, and final

polymerization for 7 minutes at 72^0C to complete elongation of all amplified strands. 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200μM dNTPs (*New England Biolab*) were used in the reaction and the amplified products were resolved on 10% non-denaturing Polyacrylamide/TBE gel. The bands were visualized after ethidium bromide staining under gel documentation system *(BioRad)*. The high annealing temperature is required in order that the primers used annealed only to CYP2D6 sequences and not to the highly homologous CYP2D7 or CYP2D8P gene.

Primer pairs CYP 1 (Exon 3: 5'- GCCTTCGCCAACCACTCCG - 3'),

CYP 2(Intron 4:5'- AAATCCTGCTCTTCCGAGGC -3')

and CYP 3 (Exon 5: 5'-GATGAGCTGCTAACTGAGCcC-3'),

CYP 4 (Intron 5:5'-CCGAGAGCATACTCGGGAC - 3')

were used to amplify regions of CYP2D6 gene over the mutations to be identified. Amplification with CYP1 and CYP2 generates a 334bp fragment which, as a consequence of G to A transition at the junction of intron 3/ exon 4, is resistant to digestion with the restriction enzyme BstN1. primer CYP3 contains a one base pair mismatch to the CYP2D6 sequence (shown in lower case) which makes the 268bp PCR fragment produced from individuals with the exon 5 mutation sensitive to digestion with the restriction enzyme *Hpa* II at the site of the base pair deletion. The other *Hpa* II site present in all individuals. Amplification conditions for each assay were as previously descried.

All restriction enzyme digests were carried out overnight using 25μL of PCR mixture and 1Unit of restriction enzyme in 1X restriction buffer concentration. For Polyacrylamide gel electrophoresis the restriction enzyme digests were mixed with an appropriate running dye (BPB). A diagrammatic representation of the expected banding patterns of amplified DNA is described in chapter 8. The digested products were resolved on 10% non-denaturing Polyacrylamide/TBE gel and visualization of the bands was done by ethidium bromide staining.

Expression of *erbB-2/Her-2 neu*, β-catenin by immunohistochemistry

Paraffin-embedded specimens of 83 cervical carcinomas cases examined microscopically, were obtained on Poly-L-Lysine coated slides. Cellular localization and expression of HER-2/neu and β-catenin was investigated by immunohistochemistry in all samples.

erbB-2 (cellular localization cell membrane) is a receptor tyrosine kinase of the *erbB* family, β-catenin is a structural component of the E-cadherin mediated cell-cell adhesion system as well as signaling molecule of the Wnt/wingless pathway.

Coating slides with Poly -L-Lysine

Poly -L-Lysine is an adhesive solution used in adhering tissue sections in glass slides, particularly useful with immunohistochemical techniques.

The Poly -L-Lysine solution Sigma (Cat # P8920) was diluted 1:10 with deionized water prior to coating slides.

The Microscope slides were washed with SDS and then dipped in 1%HCl prepared in 70% alcohol for 5' and dried to make the slides grease free before coating with Poly-L-Lysine and dipped in diluted Poly -L-Lysine solution for 5' at 18-26^{0}C. The slides were then incubated at 60^{0}C for one hour or 18-26^{0}C overnight. The coated slides can then be stored at 4^{0}C.

Immunohistochemical staining procedure

Deparaffinization

1. The slides were dipped in Xylene for 10 minutes (3 changes)
2. Then 5-10 dips were given in acetone
3. 5-10 dips in 95% ethanol 80% ethanol and 70% ethanol
4. The slides were kept in slowly running tap water for 15 minutes
5. Microwave **antigen retrieval technique** was applied, i.e. the slides were boiled resting flat with sections facing up in 10mM citrate buffer for 30 minutes using microwavable glass container, then allowed to cool to room temperature and the slides were washed briefly with Tris-HCl buffer (twice).

6. To block the **endogenous peroxidase** the slides were immersed in coplin jar containing 96mL methanol and 4mL hydrogen peroxide for 25-30 minutes at room temperature. Endogenous peroxidase blocking solution must be prepared fresh, immediately before use.
7. The slides were then washed in two changes of Tris HCl buffer for a total of around 20 minutes. Tapping off excess buffer the slides were wiped around the specimen with filter paper.
8. The slides were kept in moist chamber and the **primary antibody** was applied covering the entire specimen.
9. The slides were incubated at 4^0C overnight in the humid chamber.
10. After removing from the moist chamber the slides were washed in two changes of Tris HCl at the intervals of 10 minutes.
11. **Biotinylated secondary antibody** was applied and incubated at room temperature for one hour.
12. Again with two changes of Tris HCl the slides were washed at the interval of 10 minutes.
13. **Peroxidase conjugated streptavidin** was applied and kept at room temperature for one hour. (It is used in a labeled streptavidin-biotin immunoenzymatic antigen detection system. This technique involves the sequential incubation of the specimen with unconjugated primary antibody specific to the target antigen,

a biotinylated secondary antibody that acts with the primary antibody, enzyme labelled streptavidin and substrate-chromogen).

14. Slides were washed in two changes of Tris HCl buffer for 20 minutes.
15. **DAB** solution was prepared (immediately before use) and applied controlling the intensity under microscope using known positive control as guide. 3-3'Diaminobenzidine (DAB) is a widely used chromogen for immunohistochemical staining and immunoblotting. In the presence of peroxidase enzyme, DAB produces a brown precipitate that is insoluble in alcohol. This product is available in two component form DAB chromogen and DAB substrate. The standard working dilution is 40μL (0.9mg) of DAB chromogen per 1mL of DAB substrate. Once the two components are combined the reagent can be used for upto six hours.Combine 1-2 drops (40-100μL) of DAB chromogen with each 1mL of DAB substrate and apply mixture to the tissue section. Incubate for 5-15 minutes.
16. Immersing the slide in distilled water stopped the DAB reaction and then it was washed thoroughly (with distilled water).
17. The slides were then counterstained lightly with Harris' **hematoxylin** and dried thoroughly at room temperature.
18. Then it was mounted with coverslip using DPX, a mounting medium and labeled appropriately.

19. The slides were then observed under microscopes with positive and negative controls that were run during every batch of staining.

20. Positive control = Appropriate tissue sections known to express a particular antigen.

    Negative control = Eliminate the step of primary antibody application rest remaining unaltered.

Microsatellite Instability

5. MICROSATELLITE INSTABILITY

Introduction

Microsatellite instability (MSI) is characterized by small deletions or expansions within short tandem repeats in tumor DNA as compared with matching normal DNA. Most polymorphisms occur in the 97% of the non-coding junk DNA of the human genome, variations therein are functionally inconsequential and hence well tolerated during evolution. Much of this noncoding DNA consists of highly repetitive DNA segments, one such class in humans consists of simple tandem repeats (STRs). Such repeats of 2-3 nucleotide segments, known as microsatellite DNA are unique to each individual. All of the proteins coding regions account for only about 3% of the human genome. Most non-coding regions of DNA may be located within genes (introns) or in between the genes. Since these regions do not code for any functional protein, variations in these regions are well tolerated during evolution allowing tremendous genetic diversity to develop in these regions. Clues to the mechanism underlying the molecular basis of tumor microsatellite instability emerged from the study of the effects of mutations within the mismatch repair proteins were localized and cloned and their role in HNPCC was examined. Greater than 80% of HNPCC tumors demonstrated microsatellite instability (Aaltonen *et al.*, 1994). 30% of families with HNPCC have germline mutations in *hMSH2*, while

another 30% in *hMLH1* (Wijnen *et al.*, 1996). The presence of MSI has been reported in a variety of malignancies. Colorectal cancer was the first type of human malignancy in which MSI was described (Aaltonen et al., 1993; Thibodeau et al., 1993; Ionov et al.,1993). The reported genomic instabilities involved mono-di and tri-nucleotide repeats and occurred in hereditary as well as sporadic cancer. Tumor microsatellite instability can be detected in a PCR based assay by comparing tumor DNA to that derived from normal tissue of the same patient. Alterations of length of simple repetitive genomic sequences (microsatellite instability) characterize a distinct mechanism of carcinogenesis. MSI analysis has become an attractive method for both diagnostic and tumoribiological purposes (Dietmer et al., 1997). Instabilities in the microsatellite region of tumor DNA have been observed when compared with the normal allele of same patient showing either an expansion or contraction on one or both alleles in some tumors. Variations in such microsatellite regions are generated by errors in DNA replication. Since, microsatellite instability is one of the defects 'mismatch repair' is supposed to prevent, indicates a defective mismatch repair system.

In December 1997, the National Cancer Institute sponsored a workshop on "Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" and recommended the Bethesda Guidelines developed in 1996 to assist in selection of tumors for MSI analysis. Over the past five years the scope of MSI has been expanded to

encompass a unique form of genetic instability broadly involved in the genesis of cancer (Boland et al., 1998).

Materials and Methods

Materials and Methods used in the study have been described in Chapter 4 in detail, briefly described as follows:

Tissue Samples: Tumor biopsy specimens and corresponding peripheral blood samples (2-3mL) were obtained after appropriate consent was granted from patients who attended the cancer clinic. Clinically the majority of the tumors belonged to stage II or III, 77 tumors had moderate to well differentiated histology and 6 tumors were of poorly differentiated type.

Method: DNA was prepared from blood and biopsy samples by standard phenol chloroform methods (Sambrook et al., 1989). 40-60ng of DNA was taken as template for the PCR.

Table 5.1 Primer used in the analysis of microsatellite instability

Name	Chr	Repeat Motifs	Primer sequence	Tm (^{0}C)
BAT 25	4q	TTTT.T.TTTT$(T)_7$.A$(T)_{25}$	TCG CCT CCA AGA ATG TAA GT	58
			TCT GCA TTT TAA CTA TGG CTC	
BAT 26	2p	$(T)_5$.....$(A)_{26}$	TGA CTA CTT TTG ACT TCA GCC	58
D2S123	2p16	$(CA)_{13}TA(CA)_{15}(T/G\ A)_7$	AAC CAT TCA ACA TTT TTA ACC AAA CAG GAT GCC GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	60
APC	5q21/22	(CA)26	ACT CAC TCT AGT GAT AAA TCG`	55
(D5S346)			GC AGA TAA GAC AGT ATT ACT AGT T	
Mfd 15CA (D17S250)	17q11.2-12	$(TA)_7$....$(CA)_{24}$	GGA AGA ATC AAA TAG ACA AT GCT GGC CAT ATA TAT ATT TAA ACC	52

Polymerase Chain Reaction

A standard Polymerase chain reaction was carried out in a 25µL reaction volume containing 30-50ng of DNA as template, 20pmol of primers. One primer was end-labeled with [γ^{32}P] ATP using 0.1M DTT and incubating at 37^0C for one hour. The amplification reactions were carried out for 35 cycles at annealing temperatures ranging from 52^0-56^0C using 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200µM dNTPs (*New England Biolab*). The amplification was first checked on 2% agarose then finally resolved on 6% denaturing Polyacrylamide/ Urea gel. After autoradiography analysis of microsatellite instability was done.

MSI Analysis

MSI was characterized by small deletions or expansions within short tandem repeats in tumor DNA as compared with matching normal DNA (Fig. 5.1). Two common band patterns were observed, either a novel discrete band in tumor DNA not observed in the corresponding normal DNA or as a marked alteration of repeat length, often heterogeneous in nature and appeared as a ladder. The tumors with MSI were divided into two groups: the first is characterized by MSI-H (MSI exhibited by two or more markers) and MSI-L (MSI exhibited by one marker) and a third group lacking instability (when none of the markers exhibit instability) was MSS

In the present study, reference panel of the five markers recommended by the Bethesda Consensus Conference reference panel, were used for the first time for MSI analysis on 83 invasive squamous cell carcinoma of uterine cervix. Using this reference panel MSI-H tumors are defined as having instability in two or more markers, whereas, MSI-L tumors are defined as having instability in one marker. Microsatellite Index for the five markers [Instability observed by the primers/number of markers used] and RMA for individual primers were also calculated. [RMA = Number of cases with microsatellite instability/ total number of cases].

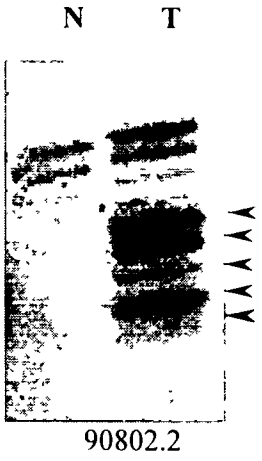


Fig. 5.1 Photograph of MSI, showing increase in the repeat units in the tumor DNA (T) (indicated by arrows), compared with the matched constitutional normal DNA (N) from the same patient.

Results

Eighty-three squamous cell carcinoma of uterine cervix were examined. Microsatellite instability [MA Index 1.0] was observed with all the five markers. Thirty-two cases were MSI-L, whereas, twelve cases showed high instability (with two or more markers). Thirty-nine cases did not show instability with any of the five markers and were therefore considered MSS. BAT25 showed maximum instability (22.9%) followed by D2S123, D17S250 and BAT 26 (20.4%, 13.25% and 9.6% respectively). D5S346 showed instability in only three cases. Representative photographs of microsatellite instability with different markers are illustrated in figure 5.3 and the results are summarized in table 5.2 Details of the patients and their respective status of MSI are presented in chart 1. Majority of the carcinoma were moderately to well differentiated type.

Table 5.2. Showing MSI in invasive cancer (MA Index = 1)

Primers	Cases	MSI	MSI (%)	RMA	LOH (%)
BAT 25	83	19/83	22.9	0.22	0
BAT 26	83	8/83	9.6	0.09	0
D2S123	83	17/83	20.4	0.20	0
D5S346	83	3/83	3.6	0.03	4.3(2/46)
D17S250	83	11/83	13.2	0.13	3.6(2/55)

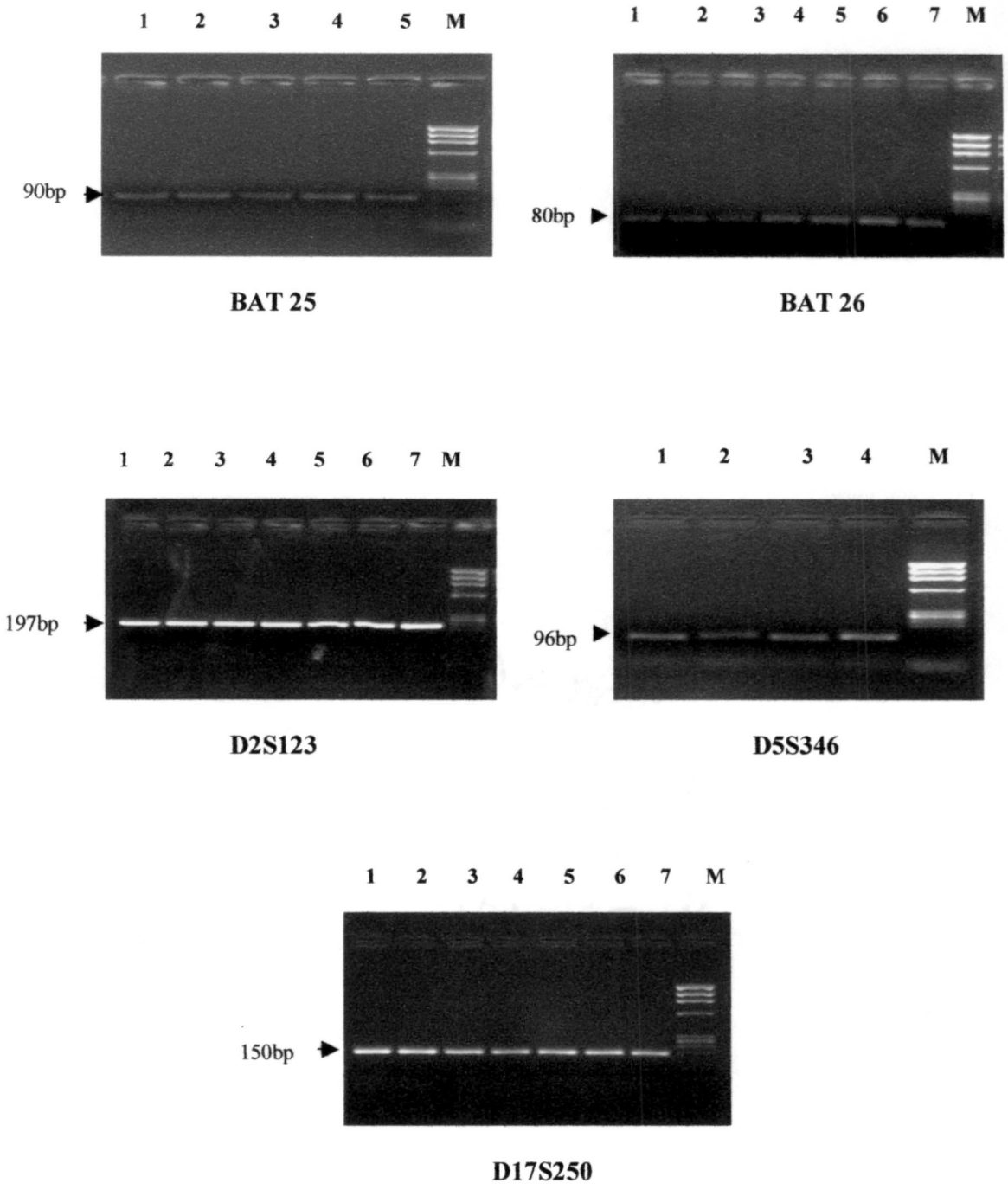


Figure 5.2 Representative photographs showing DNA amplified by five different markers used for MSI analysis and resolved on 2% agarose gels with ϕX174 Hae III digested marker. Polymorphic microsatellite markers are designated below each block.

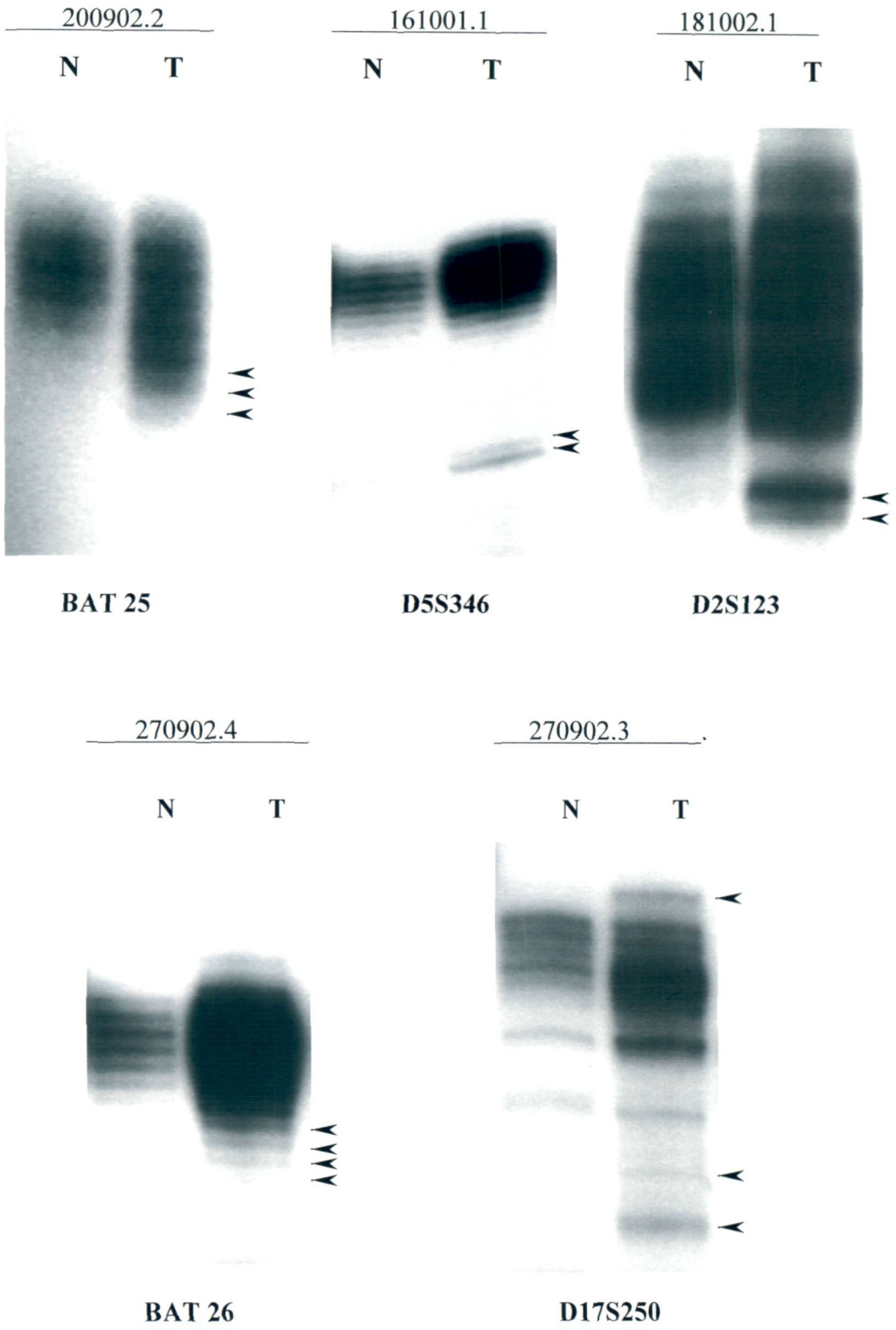


Figure 5.3 Representative autoradiograms of denaturing polyacrylamide gels demonstrating microsatellite instability (mono and di-nucleotide) using Bethesda conference reference panel of five markers [T =DNA from Tumor tissue; N= constitutional DNA from blood leukocytes of the same patient].

Discussion

Assessment of microsatellite instability (MSI) has become an important tool in tumor molecular pathology. Microsatellite instability (MSI) and microsatellite stability (MSS) appear to characterize two different pathways of carcinogenesis (Perucho,1996). Several studies have already shown that particular loci are prone to instability in different cancers; comparison between studies was difficult because of the selection of markers. In the present study we used the Bethesda Conference reference panel of five primers for the first time in cervical cancer. $(A)_n$ tracts located in the intron of *c-kit* oncogene showed maximum (22.9%) instability, followed by the dinucleotide (CA)n repeat loci D2S123 (20.4%). However, BAT-26, in intron 5 of hMSH2 gene also showed little (9.6%) instability. However, D5S346 showed 3.6% instability, which is significantly low. 13.2% instability was reported at D17S250. Our study supports the results obtained by Dietmer et al, 1997 in colorectal cancer, for which the above panel of markers was first recommended. Since, particular repeat loci are prone to instability in different cancers, Dietmer et al 1997 reported high MSI in the (A) n tracts located in the intron of c-kit oncogene (BAT-25) confirming the susceptibility of mononucleotide repeats to instability. Abnormality of the mismatch repair system has been reported in many sporadic cancers. Genetic instability was found to be 38% in gastric cancers (Han et al, 1993).

The role of MSI in invasive cervical cancer is still controversial. Chu et al (1999) and Rodriguez et al (1998) reported that incidence if MSI was as low as 5.3% and 5.6% respectively, indicating that a defect of mismatch repair system is not involved in cervical carcinogenesis. On the contrary, Wistuba et al, 1997 reported that MSI was detected in 35% invasive cervical carcinoma. Nishimura et al (2000) reported 22.9% MSI in invasive cervical cancer. Mitra et al., (1995) observed instability in 5pter region in 40% of CIS and 13% of invasive carcinomas. In the present study, MSI was present in at least one of the five loci examined (MSI-L) in 32 of 83 (38.5%) cervical carcinomas and in 12 (14%) cases. MSI was present in two or more loci (MSI-H) (chart 1). In a similar study of 100 cervical carcinomas, Chung et al, (2001), using a separate panel of five different polymorphic markers (D3S1263, D4S402, D4S415, D11S136 and D11S912) observed 25% MSI-L and 5% MSI-H.

MSI was first described in colorectal cancer (Aaltonen et al, 1993; Thibadeau et al, 1993; Ionov et al,1993). It was reported in both HNPCC as well as sporadic colorectal cancer. Subsequently MSI was described in endometrial (Risinger et al., 1993), pancreatic and gastric (Han et al., 1993), and urinary bladder cancer (Gonzalez et al., 1993). The mechanism through which MSI contributes to tumorigenesis is unknown. However, it is now widely accepted that MSI assessment should be added as a distinct criterion to refine the diagnosis of HNPCC.

The present study indicates that a panel of mono and di nucleotide repeat loci that have initially been recommended for HNPCC (Boland et al., 1998), can be used for the assessment of MSI in cervical carcinogenesis which may prove useful from a clinical diagnostic point of view.

Loss of Heterozygosity

6. LOSS OF HETEROZYGOSITY

Introduction

Loss of heterozygosity (LOH) refers to the observation of single allele at particular genetic locus or loci in benign or cancerous, or precancerous lesion, arising in an individual who is heterozygous for the same locus or loci in its normal tissue. LOH has been reported in several tumors of human origin (Yokota et al., 1989; Vogelstein et al., 1988; Wagata et al., 1991; Meltzer et al., 1987; Simon et al., 1991; Khosla et al., 1991; Fey et al., 1989; Vogelstein et al., 1989; Kovacs et al., 1988; Lee et al., 1990). Frequent LOH at a given chromosomal locus implies the existence of a tumor suppressor gene that is important in the pathogenesis of the particular cancer under study. LOH is believed to represent inactivation of one allele of a tumor suppressor gene by chromosomal or subchromosomal deletion. Frequent LOH have been detected on 3p and 5p (Kohno et al 1993; Mitra et al., 1994; Mullokandov et al 1996 and Karlsen et al 1994). Several TSGs have been reported to be located at 3p. Whereas, 5p (D5S406) showed high incidence of LOH (66.7%) in cervical cancer, suggesting the presence of candidate tumor suppressor gene (Mitra et al., 1994a). To elucidate the role of LOH at 3p and 5p regions in cervical cancer, we evaluated 83 cases of squamous cell carcinoma of uterine cervix. Clinically the majority of the tumors belonged to stage II or III, 77 tumors had

moderate to well differentiated histology and 6 tumors were of poorly differentiated type.

Materials and Methods

Materials and Methods used in the study have been described in detail in Chapter 4. Briefly described as follows.

Tissue Samples: Tumor biopsy specimens and corresponding peripheral blood samples (2-3mL) were obtained after appropriate consent was granted from patients who attended the cancer clinic.

Method: DNA was prepared from blood and biopsy samples by standard phenol chloroform methods (Sambrook et al., 1989). 40-60ng of DNA was taken as template for the PCR.

Table 6.1 Primer details for LOH analysis

MARKERS	AMPLIMER	AMPLICON SIZE
D3S1234	5'-CCT GTG AGA CAA AGC AAG AC-3' 5'-GAC ATT AGG CAC AGG GCT AA-3'	111bp
D3S1300	5'-AGC TCA CAT TCT AGT CAG CCT-3' 5'-GCC AAT TCC CCA GAT G	236bp
D3S1313	5'-CCC CTT GGA AAA TCA CTG-3' 5'-CCA TGA ATA AGC CTT GCC-3'	233bp
D5S208	5'-ACC TGA GTC TTC ATC AAT AC –3' 5'-TCC AGA ATC ATC CAT GTT GT-3'	186bp
D5S406	5'-CCT GCC AAT ACT TCA AGA AA-3' 5'-GGG ATG CTA ACT GCT GAC TA-3'	185bp

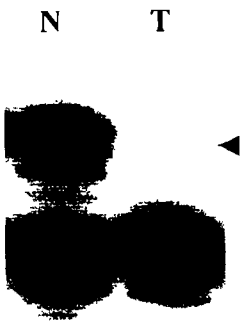
Polymerase chain reaction

Standard Polymerase chain reaction was carried out in a 25μL reaction volume containing 30-50ng of DNA as template, 20pmol of primers (table 6.1). One primer was end-labeled with [γ^{32}P] ATP using 0.1M DTT and incubating at 37^0C for one hour. The amplification reactions were carried out for 35 cycles at annealing temperatures ranging from 52^0-56^0C using 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200μM dNTPs (*New England Biolab*). The PCR products were denatured in sequencing stop buffer and subjected to electrophoresis in 6% denaturing Polyacrylamide gels, and the dried gels were autoradiographed for 6-18 hrs.

LOH Analysis

To evaluate LOH in cervical cancer, a panel of five markers was used covering the following chromosomal regions 3p14.1-14.2 (D3S1234, D3S1300, D3S1313) and 5p15.1-15.2 (D5S208 and D5S406). Markers that identified two distinguishable alleles of different sizes but similar intensity in the lane having constitutional normal DNA were termed "informative" (heterozygous). Markers that gave a single major band in the normal DNA were termed "non-informative" (homozygous). Complete absence of upper or lower allele compared with constitutional alleles was considered evidence for LOH (see fig 6.1). FRL (FRL =the number of patients with LOH in that region / the number of patients

informative in that region) was also calculated. LOH was considered significant if the frequency was above 25%.



60902.1

Fig 6.1 Representative photograph of LOH, showing complete loss of the upper allele in the tumor DNA (T) (indicated by arrow), compared with the matched constitutional normal DNA (N) from the same patient.

Results

Paired tumor/normal DNA samples from 83 SCC patients were examined for the loss of heterozygosity at five polymorphic markers mapped to chromosomal arms 3p and 5p (fig. 6.3). Frequency of LOH was high at chromosome 5p as compared to 3p, however, the difference was not significant. Highest frequency of LOH (42.6%) was observed at D5S208 followed by D5S406 (32%). 30.2% LOH was observed at D3S1234 followed by D3S1300 (27.6%) (table 6.2). Using L1 consensus primers MY09/11 in PCR amplification we found 73/83 (87.9%) of SCC to display HPV (also see chart 2). To identify high-risk types (HPV 16 and 18) PCR was done by type specific primers. 48/83

(57.8%) were HPV 16 positive. None of the cases showed positivity by HPV type 18 primers. The remaining 35 cases were positive for L1, therefore, represent HPV types, some of which may be other high-risk types. Neither HPV positivity nor presence of HPV types correlated with allelic deletions.

Table 6.2: Showing LOH in invasive cancer

Primers	No. Of Cases	Informative	LOH (%)	LOH	FRL
D3S1234	83	53	16/53	30.2	0.30
D3S1300	83	47	13/47	27.6	0.27
D3S1313	83	73	7/73	09.6	0.09
D5S208	83	54	23/54	42.6	0.42
D5S406	83	50	16/50	32.0	0.32

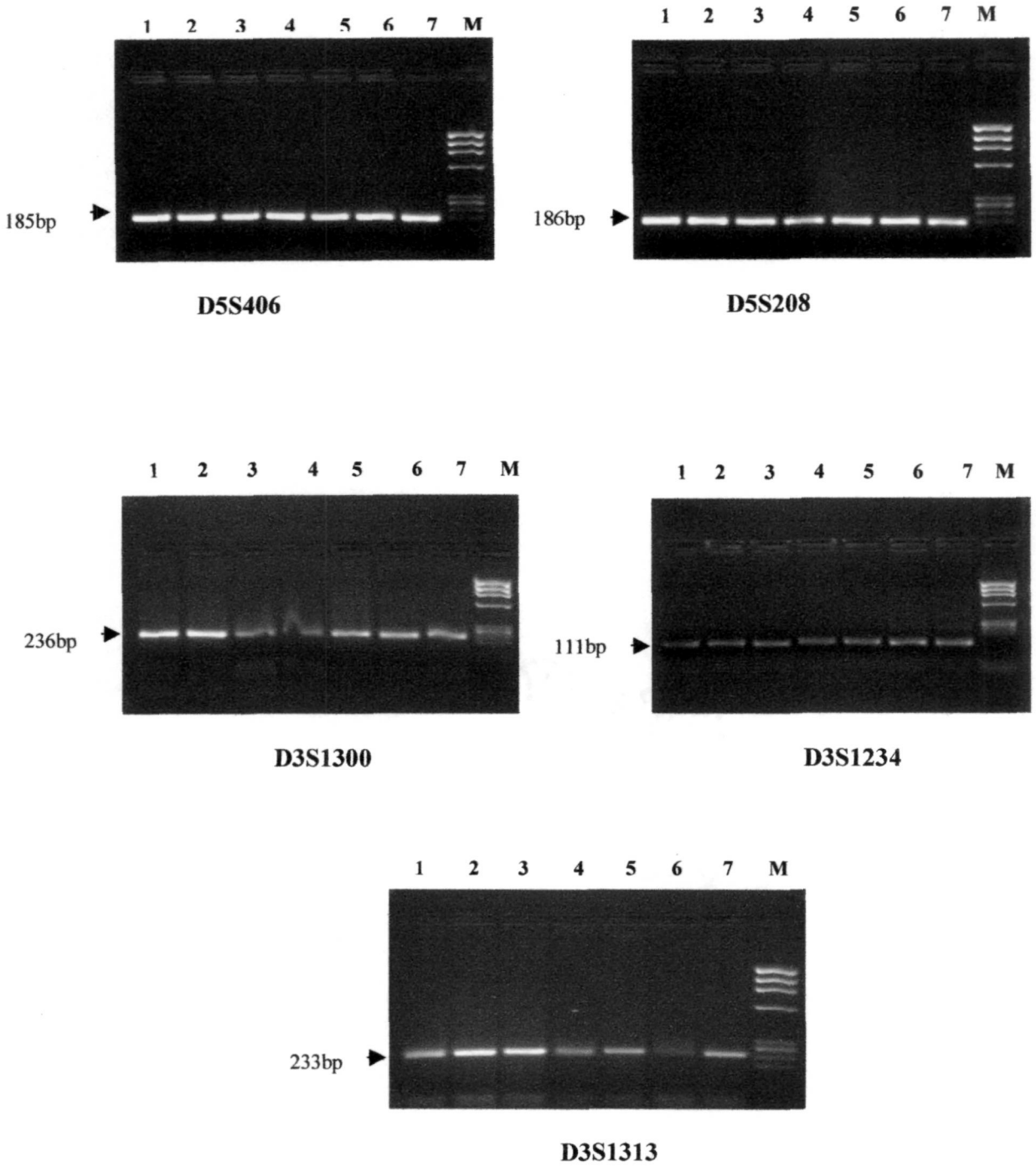


Figure 6.2 Representative photographs showing DNA amplified by five different markers used for LOH analysis and resolved on 2% agarose gels with ϕX174 Hae III digested marker.

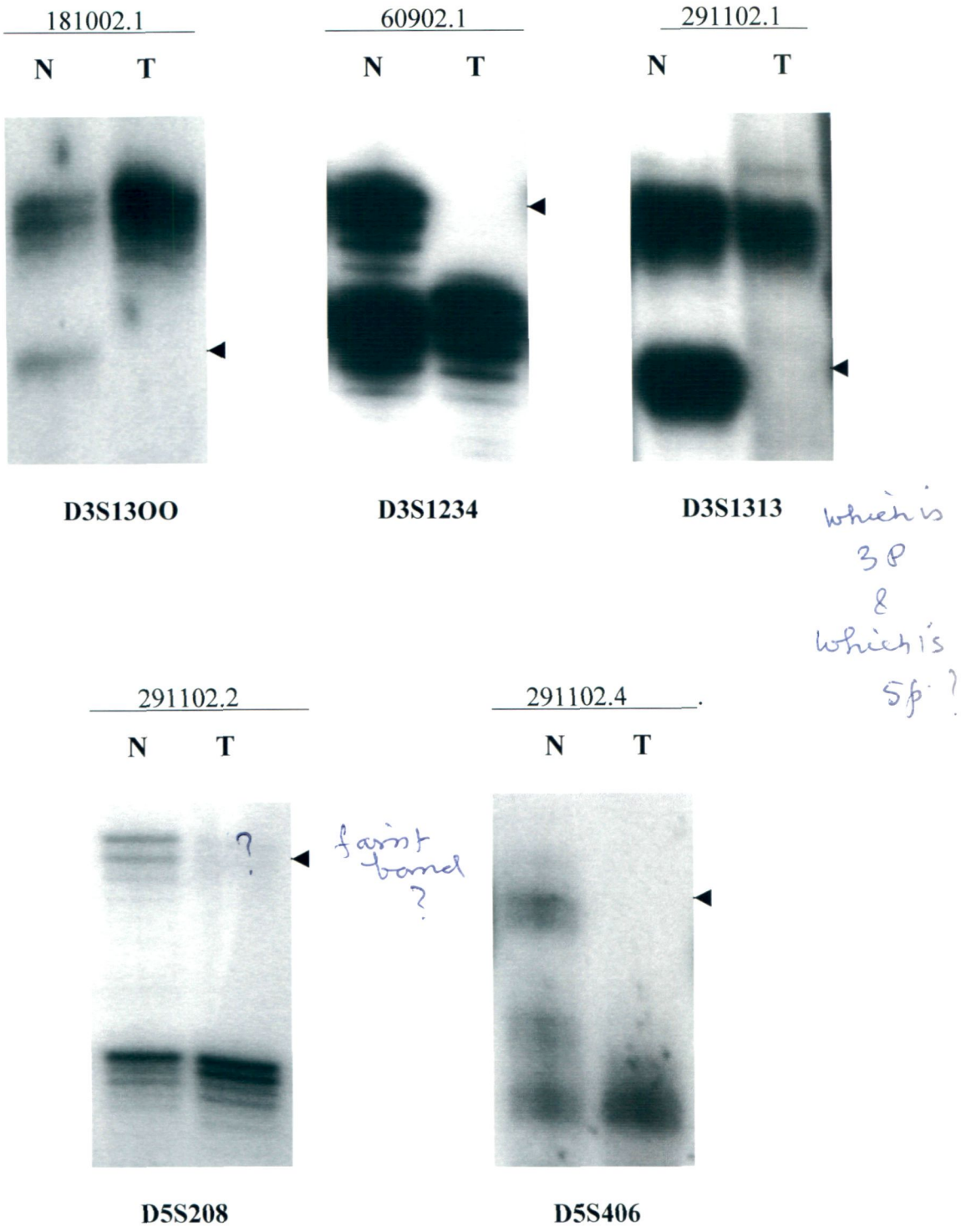


Figure 6.3 Representative autoradiograms of denaturing polyacrylamide gels demonstrating the allelic losses at chromosomal arms 3p and 5p indicated by arrows [T =DNA from Tumor tissue; N= constitutional DNA from blood leukocytes of the same patient].

Discussion

In recent studies of cytogenetics and allelotypes, many observations of allelic losses at specific chromosomal loci in a variety of human cancers have implicated the presence of TSGs on some chromosomes. In the previous study by Mitra et al., (1994a), LOH of >25% was observed at sites on eleven chromosomal arms including 3p (35%) and 5p (53%) in squamous cell carcinoma of uterine cervix.

In the present study of 83 invasive cervical cancer cases maximum frequency of LOH (42.6%) was observed at D5S208 (5p15.1-15.3) followed by D5S406 (32%) mapped to 5p15.1-15.2 Three polymorphic markers (D3S1234, D3S1300 and D3S1313) mapped to chromosome 3p14.2 region were used in the study. 3p14.2 have been reported to be frequently involved in many cancers of human origin including that of cervix. In particular deletions at 3p14.2 (FHIT gene region) are very frequent in lung cancer (Sozzi et al., 1996), breast cancer (Negrini et al., 1996), head and neck cancer (Virgilio et al., 1996), stomach and esophageal cancer (Ohta et al., 1996), pancreatic cancer (Simon et al., 1998) and cervical cancer (Greenspan et al., 1997). FHIT gene covering more than 1Mb is composed of 10 exons. The most common fragile site of human chromosome *FRA3B* also, maps within the FHIT gene. It has been speculated that fragile sites observed in the human genome correspond to chromosomal regions frequently involved in rearrangements in human cancers. A wide range 35% to 100% of

allelic imbalance frequencies at 3p loci has been reported in cervical carcinomas (Yokota et al., 1989; Chung et al., 1992; Jones and Nakamura 1992; Kohno et al., 1993; Karlsen et al., 1994; Wong et al., 1997; Chu et al., 1998; Guo et al., 1998; Kersemaekers et al., 1998a,b). Helland et al , 2000 in his study of 79 primary cervical carcinomas, using 16 markers reported 63% allelic imbalance at one or more 3p loci. The most frequently altered loci were D3S1234 (30.2%) within intron 5 of the FHIT gene followed by D3S1300 loci (27.6%). Allelotype instability at 3p14.2 suggests inactivation of the FHIT gene, which maps to this chromosomal band (Larson et al., 1997; Kohno et al., 1993; Ku et al., 1997 and Chu et al., 1998). Mitra et al., 1994a reported high frequency (53%) of LOH at D5S208 loci that maps to 5p15.1 region. In another study of recurrent cervical dysplasia (Lin et al., 2000), LOH in recurrent cervical dysplasia was reported to be significantly higher (53%) as compared to non-recurrent group (33%). LOH at 5p was the novel site reported by Mitra et al., (1994a) in cervical carcinoma. In the present study, maximum frequency of LOH (42.6%) was observed at chromosome 5p, supporting the previous results obtained by Mitra et al., (1994a), suggesting the possibility of a candidate tumor suppressor gene involved in cervical carcinogenesis. Thus, our result confirms a high frequency of allelic imbalances at chromosome arm 3p and 5p indicating the possible existence of one or more tumor suppressor genes involved

in cervical carcinogenesis. Our result also indicates the involvement of FHIT gene in cervical cancer.

Human Papillomavirus

7. HUMAN PAPILLOMAVIRUSES

Introduction

Human papillomaviruses form icosahedral non-enveloped particles with a diameter of approximately 55~~nm. They~~ contain a double stranded, circular and covalently closed DNA genome of 7500-8000bp. The virus capsid is composed of 72 capsomeres, which are arranged on an icosahedral surface lattice. Papillomaviruses have been placed as a subfamily (Shah and Howley, 1992) together with polyomaviruses in the family Papovaviridae. The circular DNA genome of papillomaviruses can be divided into three segments. The long control region (LCR), also called upstream regulatory region (URR) represents about 10% of the genome. The early (E) and late (L) genes are coded by about 50 and 40% of genome respectively. From both biological and epidemiological standpoints there are compelling evidences, which conclude that human papillomaviruses are carcinogenic in humans (IARC Working Group, 1995; Hildesheim et al.,(1994). Till date about 100 types of HPVs have been isolated and characterized. HPV DNA. More than 70 types of HPV types have been characterized so far on the basis of differences in their DNA sequences (Favre et al., 1989a,b,c and d; Heilman et al., 1980; Danos et al., 1982; Kremsdorf et al., 1983, 1984; Gismann et al., 1977; Durst et al., 1983., Boshart et al., 1984; Cole 1986; Beaudenon et al., 1986; Lorincz et al., 1987, 1989; Müller et al., 1987, Nuovo et al., 1988

etc.). Type specific prevalence is presented for the eighteen most common HPV types as identified by Clifford et al 2003; Lechner et al., 1992 in meta-analysis of HPV types in invasive cervical cancers are: HPV types 6, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, 73 and 82 in order of descending prevalence for each subgroup analysis. Two third of invasive cervical cancer cases included in the meta-analysis were associated with HPV 16 (51.0%) or 18 (16.2%) infection. Nubia Munoz et al 2003 in their epidemiologic classification of human papillomavirus types grouped fifteen HPVs as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82); three as probable high-risk types (26, 53 and 660) and 12 were classified as low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108). HPVs can induce immortalization in primary cultures of human and rodent cells. In human cells, immortalization is restricted to high-risk viruses, whereas in rodent cells, low-risk HPV types also possess weak immortalization potential. Extensive viral carcinogenesis studies the world over point to human papillomavirus virus as an important factor in cervical carcinogenesis. Of the many HPV types, types 16 and 18 are documented as high-risk HPV associated with genital neoplasia. Although a cause and effect relationship between HPV and cervical cancer is yet to be proven, over 80% of cervical cancers today are associated with HPV infection.

Materials and Methods

HPV detection was done by Polymerase chain reaction using MY09/11 consensus primers. Detection of HPV types was done using type specific primers. DNA for PCR was isolated from cervical swabs from the females using Ayer's spatula and transferred to the vials containing phosphate buffer saline. The DNA was isolated from the exfoliated cervical cells. DNA extraction method involved incubation of cell nuclei in higher concentration of Proteinase-K at 65^0C for 2.5hrs.following prolonged incubation at higher temperature, the enzyme is auto inactivated and the DNA isolated can be used directly for analysis without further purification (Gopalkrishna et al., 1992) (also see fig 7.1). 0.5-1μL of DNA was used as template for PCR.

Polymerase Chain Reaction

β-globin primers PC04 and GH20 (Saiki et al., 1988), which produces a 268bp PCR product (fig 7.2), were used as an internal amplification control. All the samples proved positive for β-globin amplification were considered for HPV analysis. DNA was amplified using MY09/11 consensus primers (Manos., et al., 1989) (figure 7.3), in thermal cycler The amplification reactions were carried out for 35 cycles at annealing temperatures ranging from 56^0 C to 58^0 C using 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200μM dNTPs (*New England Biolab*). Positive and negative controls

were also taken with every round of amplification. The PCR products were resolved on 2% agarose, stained with ethidium bromide and visualized under transilluminator or Gel Documentation system (BioRad). All the positive samples were further subjected to HPV typing with type specific primers for HPV types 6, 11, 16 and 18 (table 7.1; also see fig 7.4) or by RFLP of L1 amplicon (MY09/11) using Rsa 1 (Naqvi et al., 2004).

Table 7.1 Primers used in HPV analysis

Primer		Primer Sequence (amplimer)	Amplicon (bp)
PC04		5'-CAACTTCATCCACGTTCACC-3'	268
GH20		5'-GAAGAGCCAAGGACAGGTAC-3'	
MY09		5'- CGTCCM*ARR*GGAW*ACTGATC- 3'	~450
MY11		5'- GCMCAGGGWCATAAY*AATGG –3'	
HPV Type 6	(F)	5'- TAGTGGGCCTATGGCTCGTC -3'	280
	(R)	5'- TCCATTAGCCTCCACGGGTG -3'	
HPV Type 11	(F)	5'- GGAATACATGCGCCATGTGG-3'	360
	(R)	5'- CGAGCAGACGTCCGTCCTCG-3'	
HPV Type 16	(F)	5'- AAGGCCAACTAAATGTCAC -3'	217
	(R)	5'- CTGCTTTTATACTAACCGG -3'	
HPV Type 18	(F)	5'- ACCTTAATGAAAAACCACGA -3'	100
	(R)	5'- CGTCGTTGGAGTCGTTCCTG -3'	

**Note: M= A/C; R = A/G; W = A/T; Y =C/T*

Detection of HPV using DHCA II

Twenty-seven L1 positive cases, taken randomly, were also screened by the Digene HPV test using Hybrid Capture –II technology, which is a signal amplified hybridization antibody capture microplate assay that utilizes chemiluminiscent detection. Specimens containing the

target DNA hybridize with a specific HPV RNA probe cocktail. The resultant RNA: DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA: DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for RNA: DNA hybrids and detected with a chemiluminiscent substrate, several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrids resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured. It is a simple technique that could be readily automated for large-scale use if required. Detailed methodology is discussed in chapter 4.

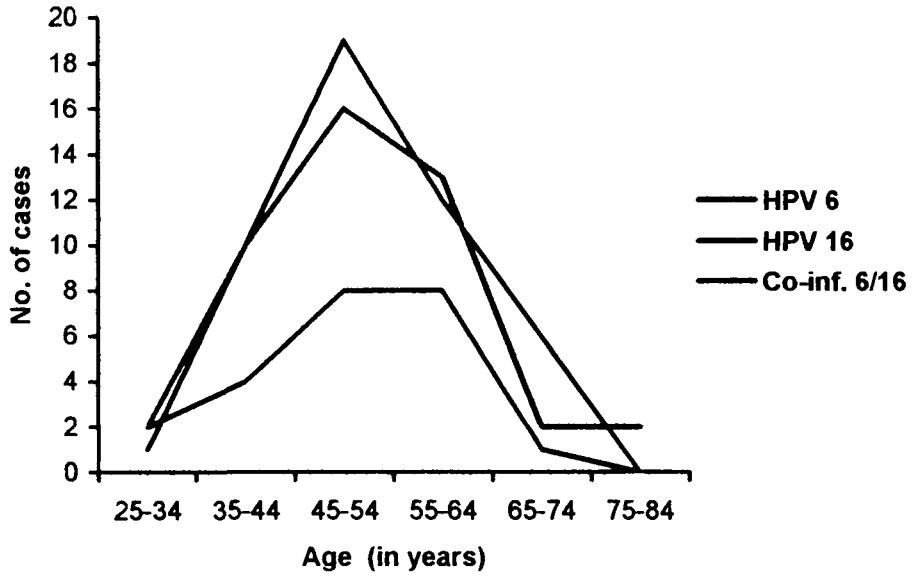
Results

All samples proved positive for β-globin amplification (Saiki et al.. 1988), the internal amplification control, and were therefore considered suitable for HPV analysis. HPV DNA was detected in 73/83 (88%) of the patients with cervical cancer using MY09/11 consensus primers of L1 region of HPV genome (Manos et al., 1989). Clinically the majority of the tumors belonged to stage II or III, 77 tumors had moderate to well differentiated histology and 6 tumors were of poorly differentiated type with median age 50 years. Further genotyping, using type specific primers for HPV types 6, 11, 16 and 18 (see table 7.1)

revealed that HPV type 16 was predominantly present 48/83 (58%), followed by type 6, 45/83 (54%) and type 11, 17/83 (20.4%), HPV type 18 was not detected in any of the samples. However, HPV type 16 was present exclusively in 20/83 (24%) cases, which was same for HPV type 6 (24%). Whereas, HPV 11 was present exclusively in 2/83 (2.4%) cases. Co-infection of HPV type 6 and 16 was present in 23/83 (28%) of cases followed by multiple infection of HPV 6 and 11 (12%).

Incidence of HPV types 6 and 16 and their co-infection with respect to age

HPV type 16 was detected in 19/48 (39%) of women of age group 45-50 years, with prevalence declining rapidly to 6/48 (12.5%) in 65 + years. Similarly, HPV type 6 showed a peak (35.5%) between age group 45-54 years and declining after 65 years (4.4%) (see graph 7.1). Interestingly co-infection of HPV types 6 and 16 showed the peak at the same age group.



Graph 7.1 Human papillomavirus types 6, 16 and their co-infection with respect to age

18/27 (67%) of the MY09/11 positive cases were found to be positive for high-risk (HR-HPV) by DHCA. Further typing of HR-HPV positive samples with type specific primers for HPV types 16 and 18 revealed that 14/18 (80%) were HPV type 16 positive while HPV type 18 was not detected in any sample, indicating the presence of few different HR-HPV types (other than types 16 and 18) in rest of the 20% samples.

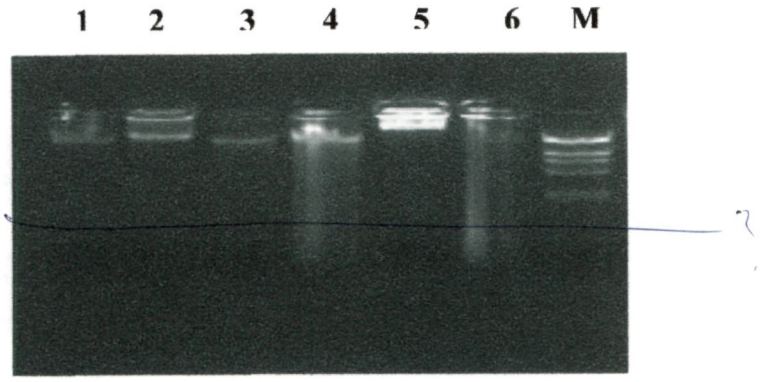


Figure 7.1 Representative photograph showing DNA resolved on 1% agarose gel [M= λ Hind III digested marker]

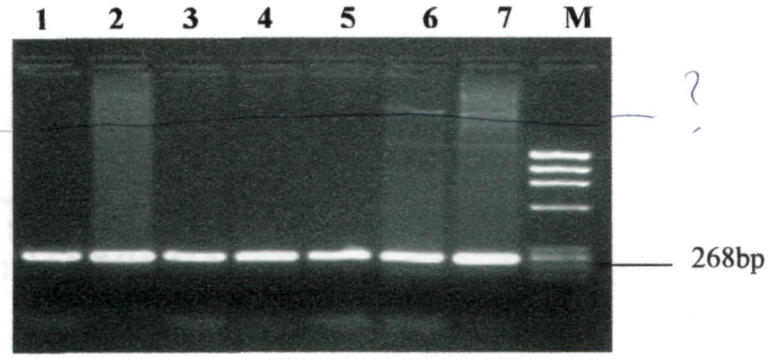


Figure 7.2 Representative photograph showing DNA amplified using beta globin primers (used as internal amplification control) resolved on 2% agarose gel [M = ϕX174 Hae III digested marker]

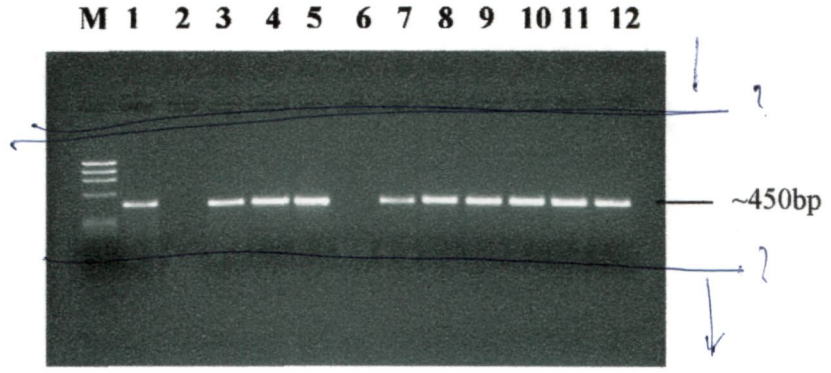


Figure 7.3 Representative photograph showing HPV positive (lanes 3,4,5&7–12) and HPV negative (lane 6) cases, amplified using MY09/11 consensus primers resolved on 2% agarose gel with positive (lane 1) and negative (lane 2) controls [M = ϕX174 Hae III digested marker]

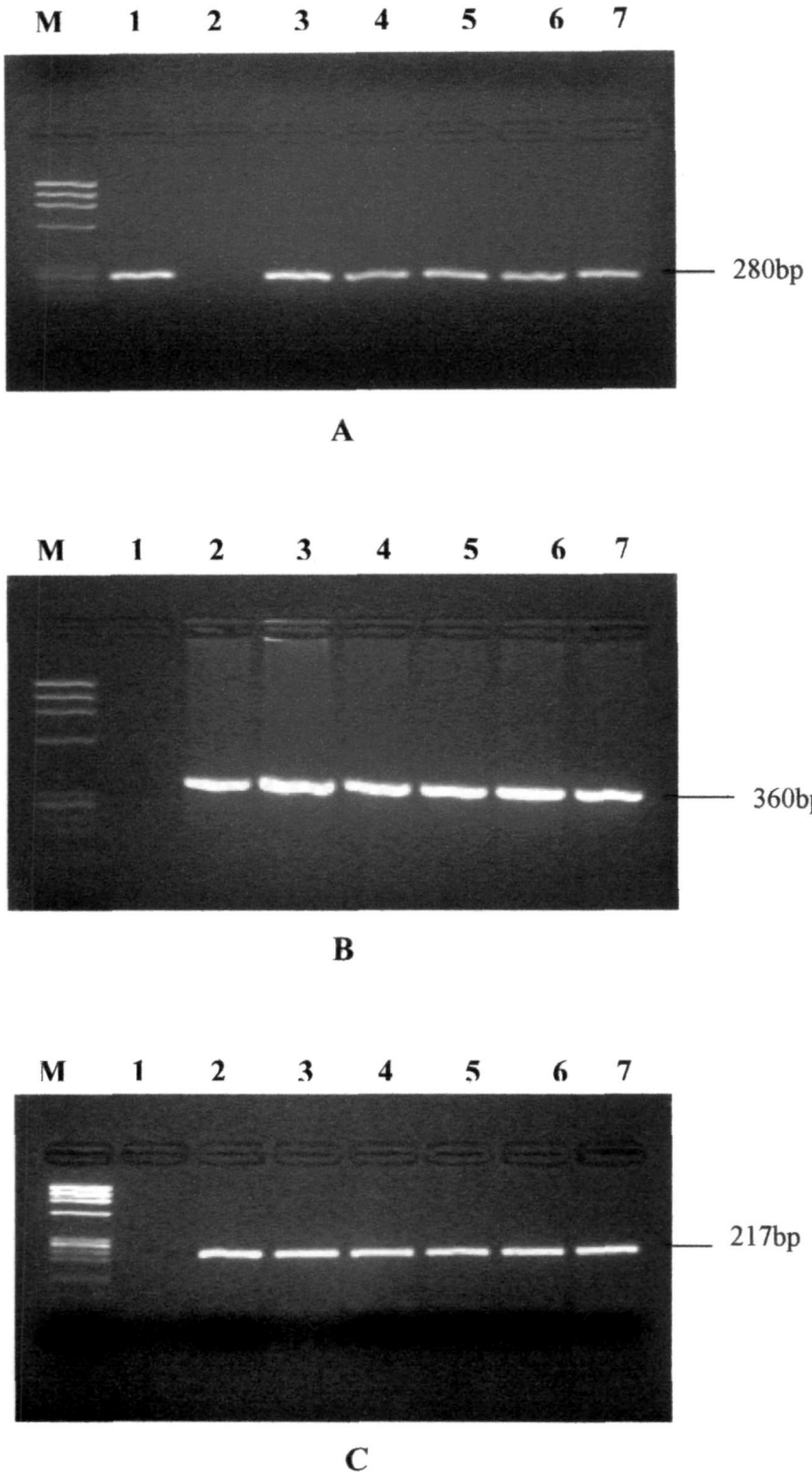


Figure 7.4 Representative photographs showing human papillomavirus type 6, 11 and 16 positive cases (A to C respectively) resolved on 2% agarose gels. Lanes 2 in B and C, lane 1 in A are positive controls and lanes 1 in B and C and lane 2 in A are negative controls. [M = ϕX174 Hae III digested marker]

Discussion

In the present study, we investigated the presence of HPV types 6, 11, 16, 18 and their co-infections in cervical cancer patients. 88% (73/83) were positive for HPV by MY09/11 consensus primers which detects a wide spectrum of both oncogenic and non-oncogenic human papillomavirus types. 27 samples from the MY09/11 positive cases were taken randomly for detection of HR-HPV types using DHCA-II kit showed 67% (18/27) positivity for HR-HPV types, of which, 80% (14/18) were HPV-16 (by PCR using type specific primers). However no HPV type 18 was detected in any of the samples. Since high-risk probe was used for the detection of HPVs by DHCA and genotyping was done by the type specific primers for HPV types 16 and 18, results suggest that HPV 16 was predominantly present among majority (80%) of the high risk types and while other high-risk types constitute only 20% (4/18) of high-risk positive samples.

All the 73 samples positive for HPV by L1 consensus primers (MY09/11) were subjected to further genotyping by type specific primers for the types 6/11/16/18 which are the most prevalent HPV types commonly associated with cervical abnormalities like warts (Schwarz et al, 1983), papillomas (Gissmann et al, 1982) and certain cancers including cancer of the uterine cervix (Bosch et al, 1995, Beaudenon et al 1986, Cole et al 1986). The high-risk viruses, such as HPV-16 (Favre et al, 1989; Seedorf et al, 1985), HPV18 (Cole ST et al,

1987) and HPV 33 (Beaudenon et al, 1986; Cole et al 1986), and few other are associated with CIN and cervical cancer. HPV 16, 18, 33 and 45 accounts for more than 90% of the high-risk types. Cervical intraepithelial neoplasia and cervical cancer both are strongly associated with persistence presence of some types of human papillomavirus. Over 90% of the cervical carcinoma have been shown to contain some high-risk HPV types (Nieminen et al, 1991). HPV 16 is considered to be prototype among malignant HPVs and HPV 11 is considered to be benign prototype (Vousden, 1993). HPV 16 has been found to be the most prevalent HPV types associated with cervical carcinoma. Earlier studies reported HPV- 16 infection in more than 80% of cancers of uterine cervix (Das et al., 1992). In the study of HPV prevalence in different countries have found the HPV-16 prevalence to be 33-50% in African countries; 34-59% in South America; 31-59% in South East Asia; 54-76.5% in Europe; 76.5% in Germany and overall percentage of HPV-16 in cervical cancer was 49.9% worldwide. Munoz et al., (2003) found HPV-16 to be present in 54.6% of cancers and HPV type 18 was present in 66% cases of adenocarcinoma. In the present study HPV 16 was found to be present in 48/83 (58%) of cancer cases. However, we observed a considerable number (54%) of HPV type 6 in cancer patients. HPV type 6 exclusively, was present in 20% of the cancer cases suggesting the possible involvement of LR-HPV types in the pathogenesis of the disease. Since, there is no clear line of demarcation

between HR and LR-HPV types. The studies show that the LR-HPV types have the capability to transactivate cells. Foster and coworkers (1994) argue that there is no linear sequence that convincingly distinguishes the high risk from low-risk HPV E6 proteins (Foster, et al., 1994). Further, binding ability of high-risk HPV's E7 for pRB complex is 10 folds higher than that of LR-HPV (Huibregste, et al., 1993). This difference results apparently from a single amino-acid modification at the position 21 (Heck, et al., 1992) affecting its oncogenicity.

In the present study HPV type11, which is mostly associated with warts, was present in 20.4% of cases. Both HPV types 6 and 16 showed peak in the age group 45-54 years and a second peak in 55-64 years of age group, which showed a declining trend after 65+ (see graph 7.1). Similar trend was observed with the co-infection of HPV types 6 and 16. Since, infection of multiple HPV DNA types is a risk factor for cervical intraepithelial neoplasia (Liaw et al., 1995), detection of LR-HPV types along with high-risk types is also of considerable importance both for diagnostic and research point of view. Serological evidences indicate protection by HPV type 6 infection against HPV type 16 cervical carcinogenesis (Sillins et al., 1999), whereas in co-infections the E6 protein of low-risk HPV (type 6) augments the ability of E7 of HPV type 16 or E7 of HPV type 6 coupled with E6 of type 16 increases the potency and ability to immortalize cells (Halbert et al., 1992). HPV type 6 was also present exclusively in 20% of the cancers, suggesting its

possible involvement in the process of carcinogenesis. These studies suggest the detection of low-risk HPV types and their co-infections along with HR-HPVs to be important for understanding the pathogenesis of the disease and the biological behavior of HPVs during cervical carcinogenesis.

CYP Polymorphism

8. Cytochrome P450 2D6 Polymorphism

Introduction

Among other environmental factors tobacco smoking is an important and frequent cause of cervical cancer. Data of cancer registry shows a positive correlation between cervical cancer and tobacco related cancers (Winkelstein, 1977). Tobacco smoke contains many potential carcinogens including polycyclic aromatic hydrocarbons, N-nitrosamines, aromatic amines, aldehydes and halomethanes. The importance of tobacco derived compounds as local carcinogens is shown by the findings that nicotine and cotinine are concentrated in cervical mucus which can be mutagenic (Winkelstein, 1990; Gram et al., 1992; Burger et al., 1993), and DNA from cervical epithelial cells of smokers contains adducts of the type expected from reaction with polycyclic aromatic hydrocarbons and aromatic amines (Simons et al., 1993). This may explain the biological plausibility of the association between smoking and cervical cancer. In addition an increased risk of cervical neoplasia and its precursors was associated with duration, intensity and early age of initiation of smoking (Harris 1980; Brinton, 1986).

The superfamily of cytochromes P450 (CYPs) consists of microsomal hemoproteins that catalyze the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds. This superfamily is divided into families and subfamilies according to homologies in their nucleic acid sequences (Nelson et al.,

1996). Cytochrome P450s constitute superfamily of enzymes crucial for the oxidative, peroxidative and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins and leukotrienes and xenobiotics including most of the therapeutic drugs and environmental pollutants (Nelson et al., 1996; Bertz and Granneman, 1997). Drug metabolism has classically been divided into "Phase I" (functionalization) and "Phase II" (conjugation) reactions. Phase I enzymes often introduce a functional group, such as hydroxyl, into the substrate; Phase II enzymes then use this functional group as a "handle" for conjugation with such moieties as glutathione, glucuronic acid, sulphate, glucose or cysteine, yielding a very hydrophilic product that can easily be excreted (Nebert et al 1990). Cytochrome P450 comprise probably more than 95% of all known Phase I enzymes, and they are important in the oxidative metabolism of numerous endogenous compounds such as steroids, fatty acids, prostaglandins, leucotrienes, and biogenic amines. Many of these enzymes also metabolize a wide range of foreign chemicals including drugs, environmental pollutants and natural plant products. P450 mediated reactions often lead to the formation of toxic metabolites, some of which may be active in tumor initiation and/or promotion (Nebert et al 1990; Meyer et al.,1990; Guengerich, 1990).

Eukaryotic cyp enzymes are membrane bound mostly localized to the endoplasmic reticulum, but some CYPs are also present in mitochondrial inner membranes. In order to function, cytochrome P450s require an electron transfer chain. In the endoplasmic reticulum this source is NADPH-cytochrome P450 reductase, previously called NADPH - cytochrome c reductase (Omura 1999). In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP (Gonzalez, 1990). Despite their occasionally minimal sequence similarity, all CYPs have a similar structural fold with a highly conserve core (Graham and Peterson, 1999).

The expression of CYP enzymes varies between individuals due to genetic and environmental factors and some diseases. These factors produce inter-individual variation in the rate and metabolic pathways of xenobiotics. One example of genetic factors influencing the inter-individual variation is the polymorphic expression of at least CYP2A6, CYP2C9, CYP2C19 and CYP2D6 among the population. The frequency of poor metabolizers (PMs) varies between races and ethnic groups. Some dietary compound, cigarette smoking, alcohol and drugs may cause induction or diminution of the expression of certain CYPs (Pelkonen and Breimer, 1994; Pelkonen et al., 1998). The human genome includes only one functional gene in CYP2D subfamily, namely CYP2D6 (Nelson et al, 1996). Cigarette smoking is a risk factor, suggesting polymorphism at loci that encode carcinogen-metabolizing

enzyme such as cytochrome P450 (CYP2D6) and glutathione S transferase (GSTT1, GSTM1) may determine susceptibility to cervical cancer. Pharmacogenetics is the study of unusual drug responses that have an heritable basis (Nebert et al., 1990). Responses can be immediate (Rash, hypotension), sub-acute (lupoid hepatitis, peripheral neuropathy), or delayed (cancer). Genetic makeup of the person dramatically influences the individual susceptibility to the adverse health effects of environmental agents.

Extensive metabolizers and Poor metabolizers:

The cytochrome P450 enzyme debrisoquine 4 hydroxylase (CYP2D6) metabolizes many drugs and carcinogens. The debrisoquine polymorphism, which is one of the most extensively studied of the P450 polymorphisms, was first discovered by R L Smith and colleagues in studies of patients taking debrisoquine, a beta adrenergic blocking agent prescribed for the treatment of hypertension. (Idle et al., 1979). Most individuals receiving the drug excrete in their urine large amounts of hydroxylated debrisoquine metabolites, and they are termed "extensive metabolizers" (EM phenotype). About 5-10% of the Caucasian population excrete the drug virtually unchanged, and they are termed "poor metabolizers"(PM phenotype) (Idle et al., 1979). The link between squamous cell carcinoma of uterine cervix and cigarette smoke, which contains carcinogenic substrates for phase I cytochrome P450 (CYP) detoxifying enzyme suggests susceptibility to this cancer may be

linked to allelic variation at this locus. CYP2D6 is a promising candidate as the PM and wild type, EM genotypes are associated with different risks of several cancers (Wolf et. al., 1992; Idle et. al., 1992).

Materials and Methods

Blood samples (5ml) were obtained from cases and control from the cancer clinic and gynecological out patient department after taking proper consent from the patients. Enzyme genotypes were determined using the Polymerase chain reaction (PCR) and restriction enzyme digestion of leukocyte DNA. As part of the medical survey all subjects were interviewed to obtain their smoking history. Smoking history was summarized as total amount of cigarettes/ *bidis* consumed during the lifetime up until the time of interview. The group comprised of females who smoked atleast 10 cigarettes/ *bidis* per day or those who have quitted smoking but have been smokers for two to three years in the past. The control group consisted of 77 females who attended gynecological out patient department having normal cervical histology (mean age 39.8 yrs) and the cases comprised of 61 mild/moderate dysplasia (mean age 33.2), 48 severe dysplasia (mean age 37.8) and 85 cases of squamous cell carcinoma (mean age 50.3) of uterine cervix.

DNA extraction from blood

DNA was extracted from the peripheral blood using standard phenol chloroform extraction method (Sambrook et al., 1989) described in detail in chapter 4.

Analysis of mutant CYP2D6 alleles by Polymerase Chain Reaction

40-60ng of DNA was taken as template and standard polymerase chain reaction was carried out in a 25μL reaction volume, with 20pmol of primers. An initial strand separation step was carried out at 94^0C (4 min), and 35 cycles of: denaturation at 94^0C for 30 sec, primer annealing at 60^0C for 1 min. and polymerization at 72^0C for 1 minute, and final polymerization for 7 minutes at 72^0C to complete elongation of all amplified strands. 1 Unit of Taq DNA Polymerase (*Roche, Applied Biosystems*), 2.5mM MgCl2 and 200μM dNTPs (*New England Biolab*) were used in the reaction and the amplified products were resolved on 10% non-denaturing Polyacrylamide/TBE gel. The bands were visualized after ethidium bromide staining under gel documentation system *(BioRad)*. The high annealing temperature is required in order that the primers used annealed only to CYP2D6 sequences and not to the highly homologous CYP2D7 or CYP2D8P gene.

Primer pairs CYP 1 (Exon 3: 5'- **GCCTTCGCCAACCACTCCG** – 3'),
CYP 2 (Intron 4: 5'- **AAATCCTGCTCTTCCGAGGC** – 3')
and CYP 3 (Exon 5:5' – **GATGAGCTGCTAACTGAGCcC**-3'),
CYP 4 (Intron 5: 5' - **CCGAGAGCATACTCGGGAC** – 3')

were used to amplify regions of CYP2D6 gene over the mutations to be identified. Amplification with CYP1 and CYP2 generates a 334bp fragment (fig 8.3A) which, as a consequence of G to A transition at the junction of intron 3/ exon 4, is resistant to digestion with the restriction enzyme BstN1, and in individuals containing db1 sequence digests 334bp fragment into fragments of 105bp and 230bp with *BstN1* (Gough et al., 1990). Primer CYP3 contains a one base pair mismatch to the CYP2D6 sequence (shown in lower case) which makes the 268bp PCR fragment (fig 8.3B) produced from individuals with the exon 5 mutation sensitive to digestion with the restriction enzyme *Hpa* II at the site of the base pair deletion. The other *Hpa* II site present in all individuals. Amplification conditions for each assay were as previously descried.

All restriction enzyme digests were carried out overnight using 25μL of PCR mixture and 1Unit of restriction enzyme in 1X restriction buffer concentration. For Polyacrylamide gel electrophoresis the restriction enzyme digests were mixed with an appropriate running dye (BPB).

A diagrammatic representation of the expected banding patterns of amplified DNA following separation of restriction fragments by polyacrylamide gel electrophoresis through a 10% non-denaturing Polyacrylamide/TBE gel is given in figures 8.1 and 8.2.

Analysis of the G to A transition

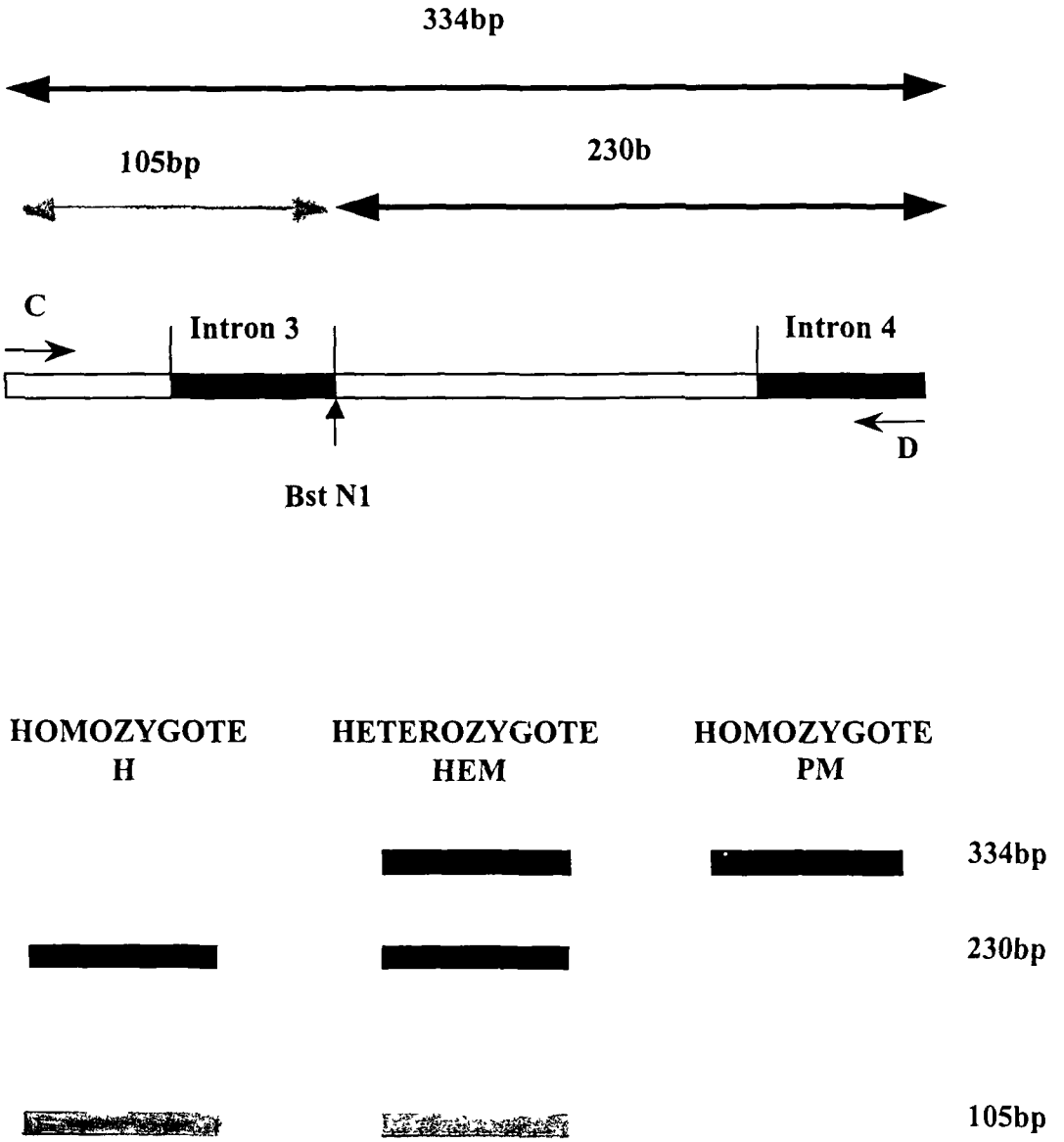
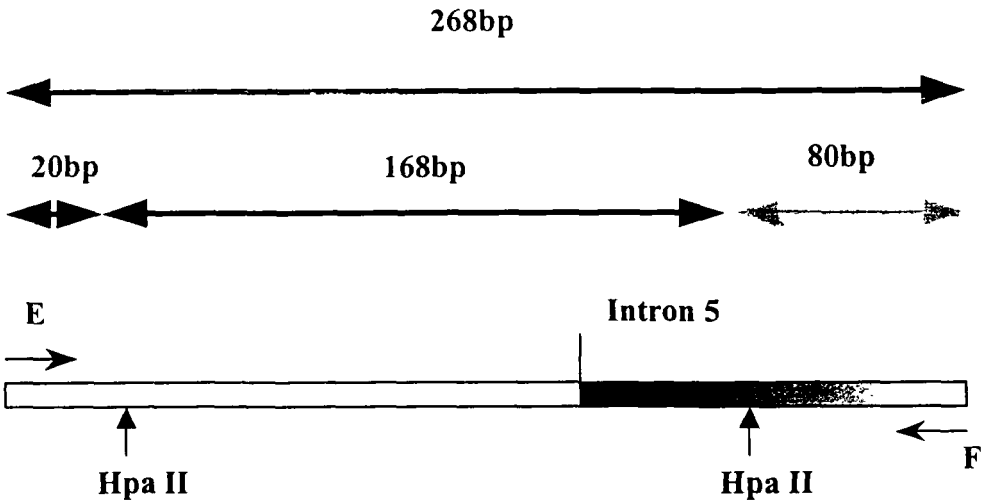


Fig. 8.1 Predicted banding patterns of digested products (*Bst NI*) of CYP 1 and CYP 2 amplicon.

Analysis of the base pair deletion



Predicted banding patterns

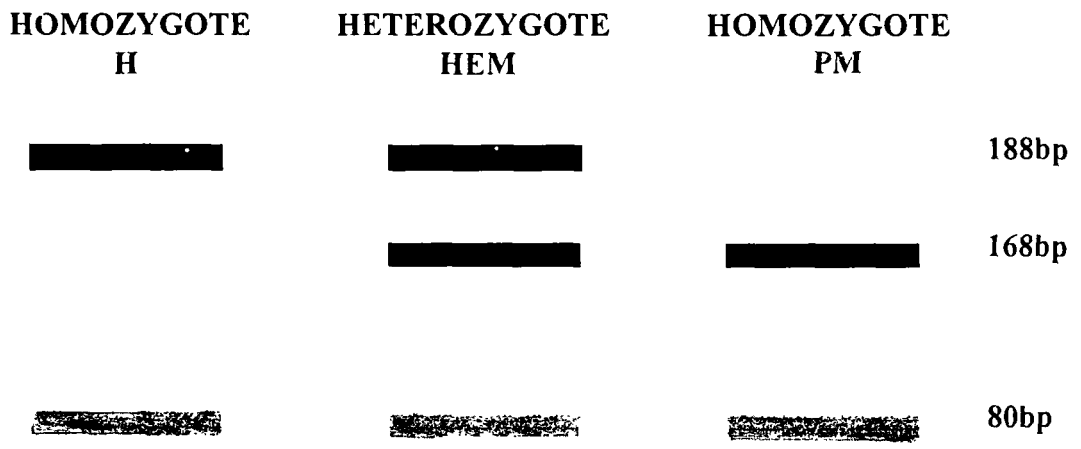


Fig. 8.2 Predicted banding patterns of digested products (*Hpa II*) of CYP 3 and CYP 4 amplicon.

Results

In the present study, the base pair deletion in exon 5 was not identified in any of the cases and majority of cases were found to be homozygous extensive metabolizers (EM). Therefore, the analysis was done on data obtained using intron3/exon4 primers (CYP1&CYP2) followed by *Bst* NI digestion (fig 8.4). Among 77 normal controls, 41 (53.2%) were homozygous extensive metabolizers (EM), 33 (42.9%) were heterozygous extensive metabolizers (HEM) and 3 (3.9%) were poor metabolizers (PM). Similarly, in 61 mild/moderate dysplasia cases 35 (57.4%) were EM, 23(37.7%) were HEM and 3(4.9%) were PM. In 48 severe dysplasia cases 29 (60.4%) were EM, 17 (35.4%) were HEM and only 2 (4.2%) were PM. In 85 cases of squamous cell carcinoma, 60 (70.6%) were EM, 19 (22.4%) were HEM and 6 (7.0%) were PM. (also see table 8.1)

The genotype frequencies at the CYP2D6 gene locus investigated in this study showed differences between cases and controls suggesting that this gene may be involved in susceptibility to cancer of uterine cervix. RFLP analysis of the CYP2D6 gene revealed a non-significant increase in the number of homozygous extensive metabolizers (EM) among the cancer patients as compared to normal controls (70.6% vs. 52.3%) (see table 8.1).

Smokers in the category of homozygous extensive metabolizers (EM) in squamous cell carcinoma were significantly high ($p<0.05$) as

compared to non-smokers. Whereas, no such correlation was observed in normal / precancerous cases. (see table 8.2)

Table 8.1 Incidence of genotypes CYP2D6 among different grades of cervical cancer

Genotypes	Normal (%)	Mild/Mod. Dysplasia (%)	Severe Dysplasia (%)	Sq. cell carcinoma (%)
EM	41/77 (53.2)	35/61 (57.4)	29/48 (60.4)	60/85 (70.6)
HEM	33/77 (42.9)	23/61 (37.7)	17/48 (35.4)	19/85 (22.4)
PM	3/77 (3.9)	3/61 (4.9)	2/48 (4.2)	6/85 (7.0)

$\chi^2 = 7.545$, $p > 0.05$, $df = 6$

Table. 8.2 Showing cases among extensive metabolizers

Category	Normal	Mild/Mod Dysplasia	Severe Dysplasia	Sq. cell carcinoma
Smokers	22	21	24	51
Non smokers	19	14	5	9

$\chi^2 = 14.79$, $p<0.05$

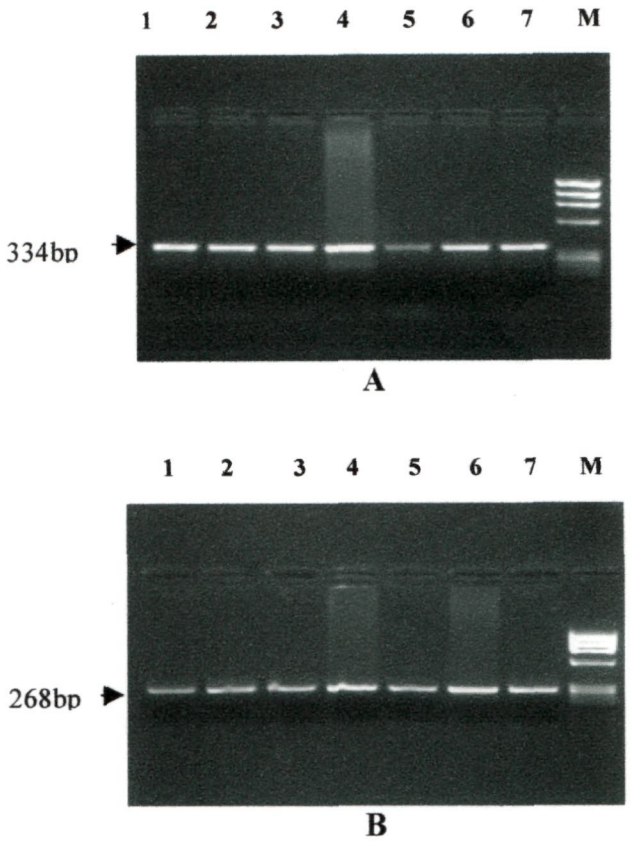


Figure 8.3 Representative photographs showing DNA amplified using primers CYP 1 & 2 (A, 334bp), CYP 3 & 4 (B, 268bp), resolved on 2% agarose gel with ϕX 174 (Hae III digested marker)

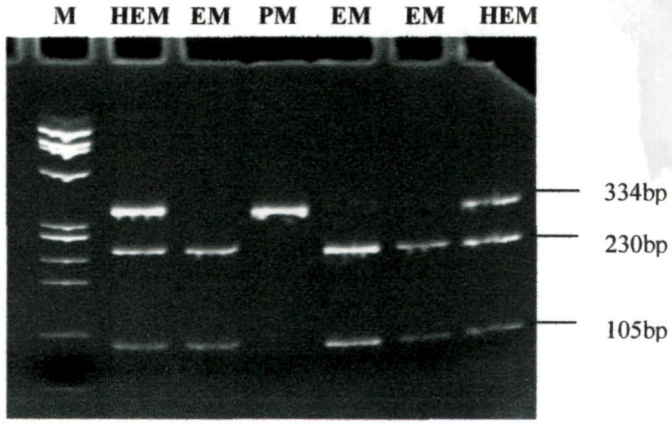


Figure 8.4 Representative photograph showing digested products of 334bp amplicons from exon 3 to intron 4 (CYP 1 & 2, *Bst NI*) resolved on 10% non denaturing polyacrylamide gel. [M = ϕX174 Hae III digested marker; EM = homozygote extensive metabolizer; HEM = heterozygote extensive metabolizer and PM = poor metabolizer]

very nice gel picture!!

Discussion

The CYP2D6 polymorphisms have been associated with an increase risk for smoking related cancers such as lung, bladder and head and neck cancer (Nakachi et al., 1993; Kihara et al., 1994; Trinza et al., 1995; Park et al., 1997; Mc Williams et al., 1996; Bell et al., 1993; Muscat et al., 1996). Cigarette smoking is a risk factor for cervical neoplasia (Winkelstein, 1990). In the present study of 77 normal and 194 cases comprising 61 mild/moderate dysplasia, 48 severe dysplasia and 85 squamous cell carcinoma, a significant association between smoking and CYP2D6 EM genotype in cancer cases was observed. However, no significant difference was observed in precancers.

Cytochrome P450 activity is polymorphic and has also been linked to lung cancer risk (Ayesh et al., 1984; Caporaso et al., 1990). CYP2D6 hydroxylates xenobiotics such as debrisoquine, and a tobacco specific N-nitrosamine (Gonzalez et al., 1990) and an individual's polymorphic phenotype is inherited as an autosomal recessive manner.

There are two possible explanations for the association of smoking with cervical neoplasia. Firstly, chemical carcinogens in tobacco smoke might directly induce cervical carcinogenesis; secondly, cigarette smoking may produce a local immunologic defect. Smoking is associated with a significant decrease in the concentration of antigen presenting Langerhans' cell in the normal cervical epithelium (Barton et al., 1988). In the present study we described that the susceptibility to

squamous cell carcinoma is influenced by polymorphisms at CYP2D6. Our data suggests that CYP2D6 and smoking is a high-risk combination, which categorizes women susceptible to squamous cell carcinoma. Since, several genotypes have been associated with cancer risk (Idle et al , 1992; Wolf et al.. 1992: Nakachi et al., 1993). These include the CYP2D6 poor metabolizer (PM) genotype that results from several gene inactivating mutations (Gough et al., 1990; Wolf et al., 1992). Significantly, the PM genotype may also enhance risk, thus, studies in leukemia and malignant melanoma show increased frequency of mutant alleles implicating impaired detoxification of an unidentified chemicals (Wolf et al., 1992). A significant influence of CYP2D6 and susceptibility to cervical intraepithelial neoplasia was observed in women who smoked (Warwick et al., 1994).

The method used in the present study for genotype analysis of the CYP2D6 polymorphism allowed us to identify the wild type homozygous extensive metabolizers (EM), the heterozygous extensive metabolizers (HEM) and poor metabolizers (PM) unequivocally. Our results indicate an increase in the number of homozygous EM in cervical carcinoma patients relative to the controls. Warwick et al., (1994) in the study of cervical intraepithelial neoplasia and CYP2D6 polymorphism suggested that CYP2D6 EM smokers have increased susceptibility to high grade CIN but are less likely to progress to squamous cell carcinoma, whereas, our results support the study of Simons et al., (1993) showing smokers to have increased susceptibility to cervical cancer.

Expression of Oncogenes

9. Expression of erbB-2 and Beta catenin in cervical cancer

Introduction

Activation of various oncogenes has been reported to be involved in the development of several human cancers. More than twenty such oncogenes have been detected in different human cancers, acting on the similar pathway of cell proliferation. These genes were first identified from oncogenic RNA viruses called retroviruses. These oncogenes are named after the viral strain from which they were isolated: e.g. *rel, src, myb, erb*-A, *erb*-B, *fps, yes, ros, mos, ras, abl, fes, fms and sis.* Recently, amplification of cellular proto-oncogenes has been found to be associated with tumor progression (Bishop et al., 1991) e.g. *MYCN* in neuroblastomas (Brodeur et al., 1984), small cell lung cancer (Wong et al. 1986) and various other tumor types; and *erb*B-2 (HER-2/nue) in breast (Slamon et al., 1987; Skalova et al.2003) ovarian (Slamon et al., 1989; Tyson et al., 1991; Hou et al., 1996), gastric (Houldsworth et al., 1990; Al-Kasspooles et al., 1993) and bladder carcinomas (Coombs et. al., 1991) suggesting that this gene may play a role in tumorigenesis (Bishop et al., 1991). Expression of oncogenes, including *ras, myc* and *erbB-2* has been reported in a high proportion of cholangiocarcinomas. The oncogene *erbB-2* located on chromosome 17q has been reported to be frequently amplified and overexpressed in many adenocarcinomas and carcinoma of breast (Slamon et al., 1989), stomach (Yokota et al.,

1988), renal (Freeman et al., 1989) and ovary (Slamon et al., 1989; Tyson, et al., 1991; Hou et al., 1996). The *erb*B-2 oncogene has been found to be a truncated version of the gene coding for epidermal growth factor receptor. The tyrosine kinase domain in the viral *erb*B-2 oncogene is intact but its catalytic activity is increased because of a shorter extra cellular domain. However, expression of genes may occur by various pathways like gene mutation, amplification etc.

In a comprehensive study Mitra et al., (1994b) studied 22 proto-oncogenes for amplification in 50 primary untreated squamous cell carcinoma of uterine cervix belonging to clinical stages II and III and histologically all moderately to well differentiated, high amplification of *erb*B-2 was observed. Further, Sharma et al., (1999) reported frequent amplification of *erbB-2 (HER-2/neu)* oncogene in cervical carcinoma using non-fluorescence in-situ hybridization technique on paraffin sections. Overexpression of *erb*B-2 protein is associated with amplification of *erbB-2* gene and frequently occurred in cervical adenocarcinomas of patients with poor prognosis (Kihana et al., 1994). Human epidermal growth factor receptor-2 (HER-2/neu; erbB-2) belongs to a family of four transmembrane receptor tyrosine kinases involved in signal transduction pathways that regulate cell growth and proliferation. Amplification or overexpression of HER-2/neu occurs in about 30% of human breast and ovarian cancers and is associated with a poor clinical outcome, including short survival time and short time to

relapse. Recent advances in our understanding of HER-2/neu signaling pathways have greatly increased our knowledge of breast cancer tumorigenesis and have provided new targets for treating breast and ovarian tumors that overexpress HER-2/neu. Although controversial still many studies support that *HER-2/neu* overexpression leads to a high degree of malignancy and may predict a poor prognosis and shorter survival of the patients. The erbB-2 (HER-2/neu) oncogene protein is found over-expressed in 20-30% of all breast cancers. However, <20% of tumors with high erbB-2 levels show objective response to the anti-erbB-2 targeted therapeutic, Trastuzumab (Herceptin). Herceptin is the first therapy for breast cancer that targets an oncogene product. Thus, the ways in which erbB-2 stimulates progression and maintenance of tumor growth is highly complex and incompletely understood. The recombinant humanized anti-erbB-2/HER-2/neu monoclonal antibody Herceptin (Trastuzumab) has been shown to significantly enhance the tumoricidal effects of antitumor drugs such as paclitaxel (Taxol) in patients with erbB-2 overexpressing breast cancers. Several studies suggest that Herceptin enhances the antitumor effects of Taxol. Herceptin pretreatment renders *erbB-2* overexpressing breast cancers more susceptible to Taxol-induced cell death, which may have important clinical therapeutic implications.

It has been further revealed that beta-catenin, a central component of the cadherin adhesion system, binds to both the

cytoplasmic domain of cadherin and the amino-terminal domain of α-catenin during cell adhesion, mediates the interactions of the cadherin catenin complex with the erbB-2 gene product and epidermal growth factor receptor (Hoschuetzky et al., 1994; Ochiai A., et al., 1994; Kanai et al., 1995). E-cadherin is suggested to be the major cell adhesion molecule in the mammary gland, in the cytoplasm E-cadherin is linked to beta-catenin and alpha-catenin, which mediate the connection of the cytoskeleton. Further, in invasive ductal carcinomas using immuno-histochemistry, disruption of this cell adhesion system by erbB-2 oncoprotein through beta-catenin phosphorylation was observed (Nagae et al., 2002). These results suggest that the E-cadherin-mediated cell adhesion system is frequently lost in invasive ductal-type breast cancers by random loss of E-cadherin/catenins or erbB-2 overexpression, and that the preservation of this system correlates with well-differentiated morphological features.

Ougolkov et al., (2000) in early gastric cancer reported 12 out of 14 cases with erbB-2 overexpression also showing altered beta-catenin expression, suggesting that both molecules are involved in the development of a certain set of differentiated early gastric cancers (EGCs). However no such information is available in cervical cancer.

To understand the extent of involvement of erbB-2 and beta catenin in the development of cervical cancer the study has been planned

to know the state of expression of erbB-2 and beta catenin in different stages of cancerous lesions of uterine cervix.

The accumulation of cytoplasmic *β-catenin* is reported to result from the genetic mutation of APC and Axin (Ikeda et al., 1998) or that of β-catenin itself (Munemitsu et al., 1995) Thus overexpression of beta-catenin in the cytoplasm or nucleus is often found in cancers of various organs, including the large intestine (Takayama et al., 1998), endometrium, ovaries (Davies et al., 1998), esophagus (Yutaka Kimura et al., 1999), thyroid (Huang et al., 1998), soft tissue (Alman et al., 1997) and liver (Nhieu et al.,1999). Therefore the localization (cell membrane, nuclei or cytoplasm) of *β-catenin* is of considerable importance. *β-catenin* (95kDa) is considered to mediate interaction between E-cadherin and α-*catenin* as *β-catenin* binds with both the cytoplasmic domain of E-cadherin and *α-catenin. β-catenin* has two distinct roles in E-cadherin mediated cell adhesion and carcinogenesis through APC gene mutation. One occurs at cell adhesion sites where cadherins become linked to the actin based cytoskeleton, the other occurs in the cytoplasm and nuclei and are thought to regulate cell transformation.

In order to evaluate the role of erbB-2 and *β-catenin* in the development of cervical cancer, we studied expression of both cytoplasmic and membranous *β-catenin* in the cancerous lesions of

uterine cervix in relation to HPV infection, which is considered to have strong association with this cancer.

Materials and Methods

In the present study fifty cervical cancer cases (including 28 squamous cell carcinoma and 22 adenocarcinoma) were examined. Lesions were graded histologically according to the World Health Organization classification (Watanabe et al., 1990). Paraffin sections (5μ thick) were obtained on Poly-L-Lysine coated slides. Cellular localization and expression of *erbB-2 (HER-2/neu)* and *β-catenin* was investigated by immunohistochemistry. 3'Diaminobenzidine (DAB) was used as chromogen for staining. Primary antibodies, biotinylated secondary antibody and peroxidase-conjugated streptavidin were obtained from Neomarker [LSAB Kit, (Ready to use)].

Level of expression of erbB-2 protein was classified as follows: *Negative* (-): the cells were not stained at all or the cytoplasm of less than half the cells was weakly and diffusely stained; *weakly positive* (+): the cytoplasm of at least half of the cells was weakly and diffusely stained; and *strongly positive* (++): the cell membrane was definitely stained in all or a substantial portion of the cancer cells (greater than 20%).

Evaluation of immunostaining for the expression of *beta catenin* was done by comparing the intensity of staining in the normal epithelial

cells and the tumor cells. If the intensity in tumor cells was the same as in normal epithelial cells or cancer cells express *β-catenin* on the cell-cell boundaries same as normal epithelial cells, the tumor cells were evaluated as preserved expression (+) (also see charts 3 & 4). Cases in which the intensity of staining was homogenously weak or variable, or the tumor cells showed diffuse or obscured staining in the cytoplasm, the cancerous tissue was evaluated as reduced expression (±) (see charts 3 & 4) (Takayama et al., 1996). Assessment of the staining was carried out using light microscopy and was confirmed by at least two independent observers with no knowledge of histological grade and patients history. Two observers scored staining independently and a high level of concordance (95%) was achieved. Chi-square test and Fisher exact test were used as per requirement.

Results

Fifty cases, (including 28 squamous cell carcinoma, 22 adenocarcinoma) and eleven controls (females with chronic cervicitis), were investigated immunohistochemically to see the expression of *erbB-2* protein. The squamous cell carcinoma cases included 3 poorly differentiated, 13 moderately differentiated and 12 well differentiated squamous cell carcinoma. One out of eleven controls showed *erbB-2* expression whereas, *erbB-2* expression was significantly high ($p<0.05$) in squamous cell carcinoma (Figures 9.2, 9.3 and 9.4) and was found to be 32.1% (9/28); of which, 66.6% (6/9) were strongly positive (++) for

erbB2 expression and in 33.3% (3/9) *erbB-2* expression was weakly positive (+) (see chart 3). Strong correlation ($p<0.05$) between erbB-2 expression and human papillomavirus type 16 infection was observed in squamous cell carcinoma, 88.9% (8/9) of the cases showing *erbB-2* expression (+/++) were positive for HPV type 16, whereas, *erbB-2* expression in HPV 16 negative cases was 11.1%. Further, 33.3% (4/12) of well differentiated squamous cell carcinoma showed *erbB-2* expression, of which all (100%) were positive for HPV type 16, similarly 38.4% (5/13) of moderately differentiated squamous cell carcinoma showed *erbB-2* expression of which 80% (4/5) were HPV type 16 positive, whereas there was no *erbB-2* expression among the poorly differentiated squamous cell carcinomas, however, all were HPV positive but none was positive for HPV type 16. Among 22 adenocarcinoma (Figures 9.6 and 9.7) of uterine cervix and 7 controls, expression of *erbB-2* oncogene was found to be 31.8% (7/22) in adenocarcinoma (chart 4), whereas, none of the controls showed *erbB-2* expression.

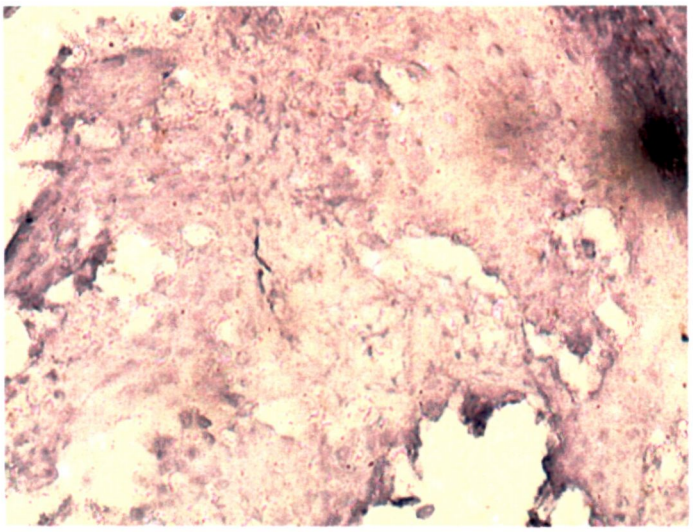
Table 9.1 Showing *erb*B-2 and *β-catenin* expression in squamous cell carcinoma and adenocarcinoma of the uterine cervix

Histology		*erbB-2*			*beta catenin*	
		(-)	(+)	(++)	+	±
Squamous cell Carcinoma (SCC) (n=28)	Poorly diff. SCC	3/3	0/3	0/3	3/3	0/3
	Moderately diff. SCC	8/13	2/13	3/13	7/13	6/13
	Well diff. SCC	8/12	1/12	3/12	4/12	8/12
Adenocarcinoma (n=22)		15/22	5/22	2/22	15/22	7/22

Expression of *β-catenin* was immunohistochemically investigated in fifty cases, including 28 squamous cell carcinoma (13 moderately differentiated, 12 well differentiated and 3 poorly differentiated carcinoma) and 22 adenocarcinoma, and eleven controls (females with chronic cervicitis). The membranous staining observed in healthy specimens was used as control (Fig. 9.8A). Out of the 28 squamous cell carcinoma of uterine cervix, 50% (14/28) were evaluated as *beta-catenin* preserved and 50% were evaluated as reduced expression. In the tumors with preserved β-*catenin* expression, cells expressed β-*catenin* on the cell-cell boundaries same as normal epithelial cells (Fig 9.8A). On the other hand in tumors with reduced *beta-catenin* expression, cells showed obscure or diffused staining on the membrane and increased cytoplasmic or nuclear staining (Fig 9.8B). In moderately differentiated squamous

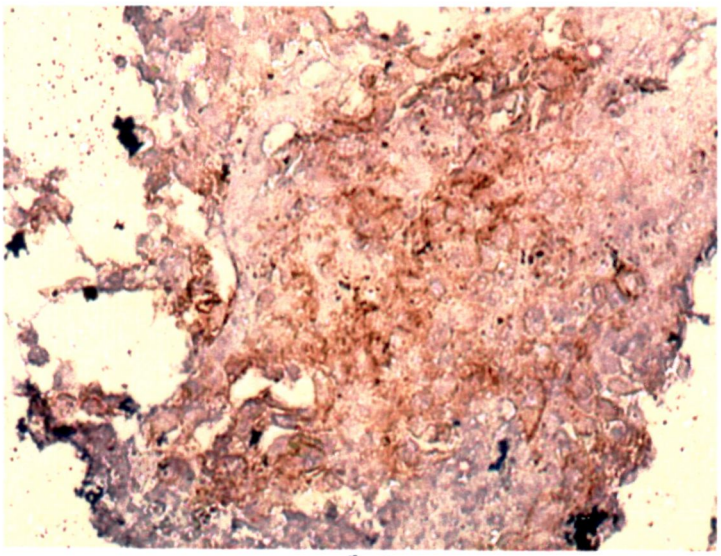
cell carcinoma reduced beta catenin expression was observed in 46% (6/13) cases, while in well differentiated squamous cell carcinoma reduced *beta* catenin expression was observed in 66.6% (8/12) cases. However, none of the poorly differentiated squamous cell carcinoma showed reduced expression of *beta-catenin.* Further, reduced beta catenin expression was found to be high among HPV type 16 positive cases. Five out of eight (62.5%) of well-differentiated tumors with reduced *beta-catenin* expression were HPV 16 positive, similarly 3/6 (50%) of moderately differentiated tumors with reduced *beta-catenin* expression were positive for HPV type 16. Further, *erbB-2* expression among cases with reduced beta-catenin expression was significantly higher ($p<0.05$) as compared to that in preserved beta-catenin expression. Out of 14 cases showing reduced *beta-catenin* expression, 7(50%) were erbB-2 positive whereas, only 2/14 (14.3%) tumors with preserved β-*catenin* expression were *erbB*-2 positive. Thus, in squamous cell carcinoma, erbB-2 expression in cases with reduced *beta catenin* expression was significantly higher ($p<0.05$) as compared to those with preserved *beta catenin* expression. However, no such correlation between *beta catenin* and *erbB*-2 overexpression was observed in adenocarcinoma.

Analysis of Her-2 neu expression by immunohistochemistry performed on formalin fixed, paraffin embedded sections of cervical lesions displaying various levels of aggressiveness



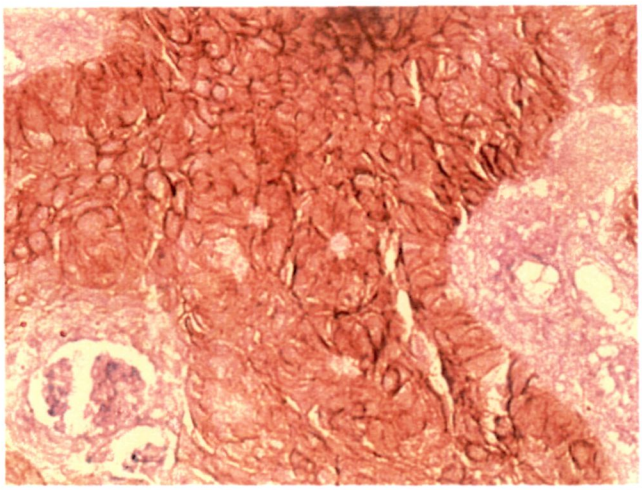
9.1

Fig. Negative control (showing absence of immunoreactivity of Her-2 neu due to elimination of primary antibody)



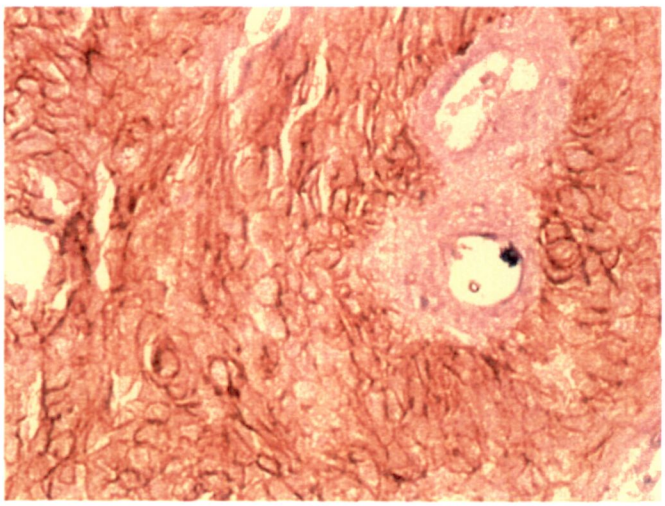
9.2

Fig Poorly differentiated squamous cell carcinoma



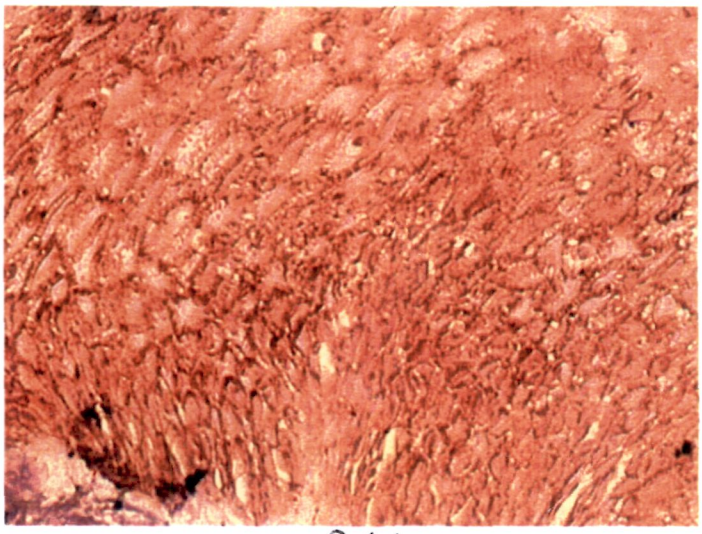
9.3A

Fig Moderately differentiated squamous cell carcinoma



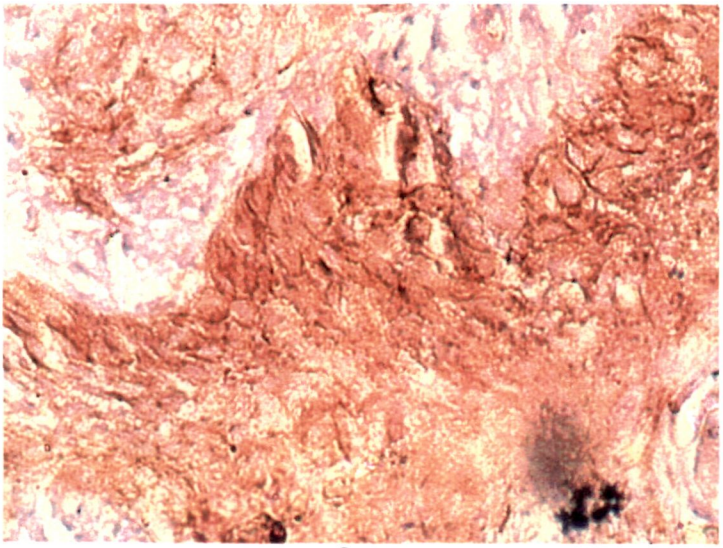
9.3B

Fig Moderately differentiated squamous cell carcinoma



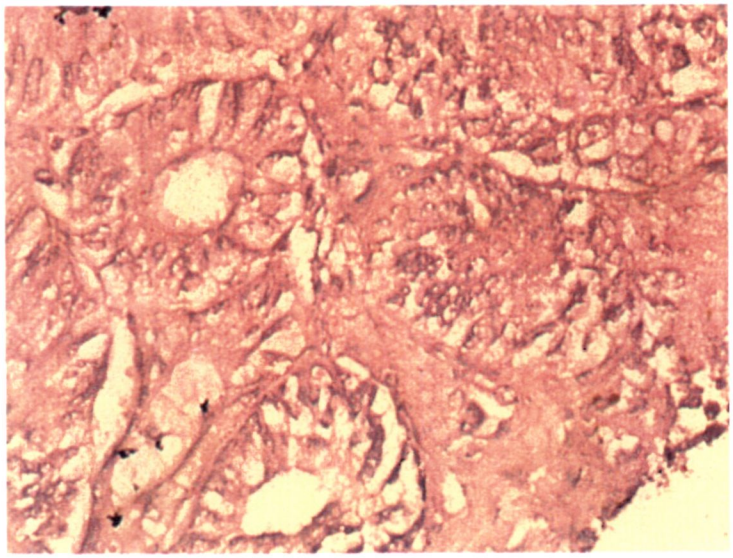
9·4A

Fig Well differentiated squamous cell carcinoma

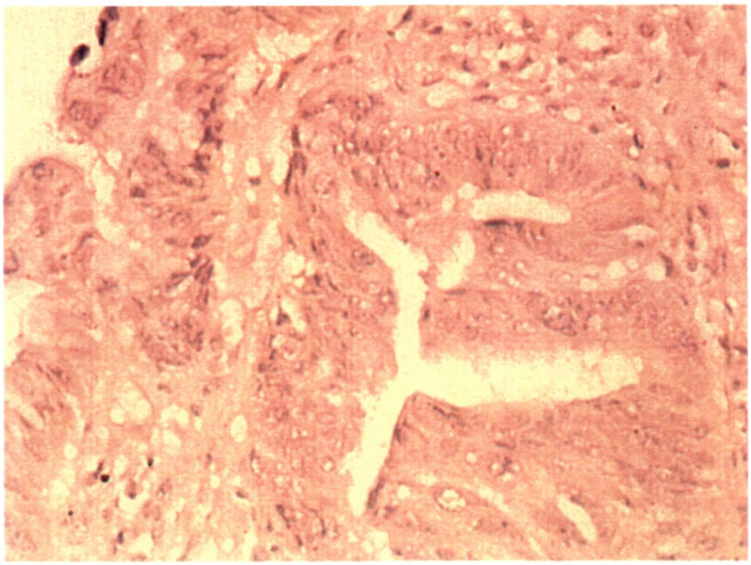


9·4B

Fig Well differentiated squamous cell carcinoma

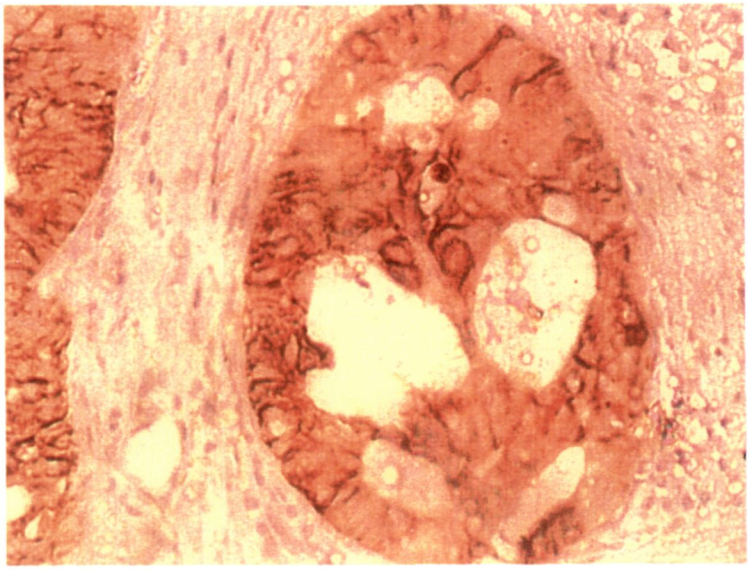


9.5 A

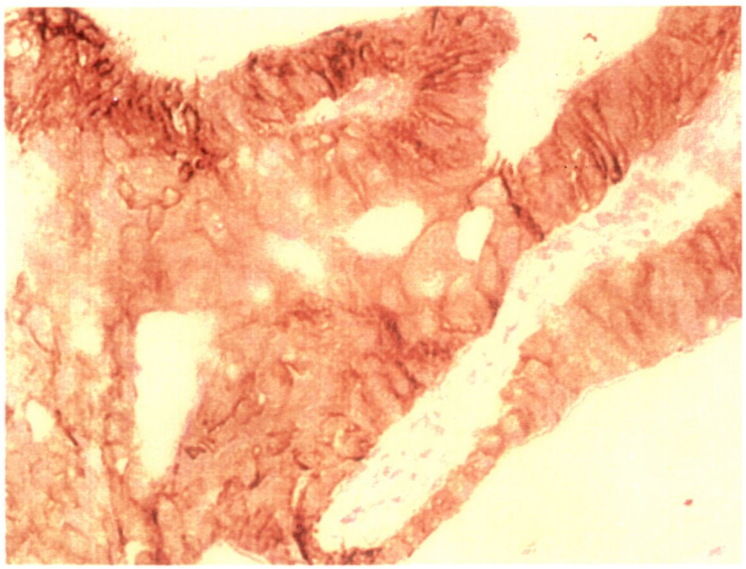


9.5 B

Fig. Negative controls (A & B) showing absence of immunoreactivity of Her-2 neu due to elimination of primary antibody

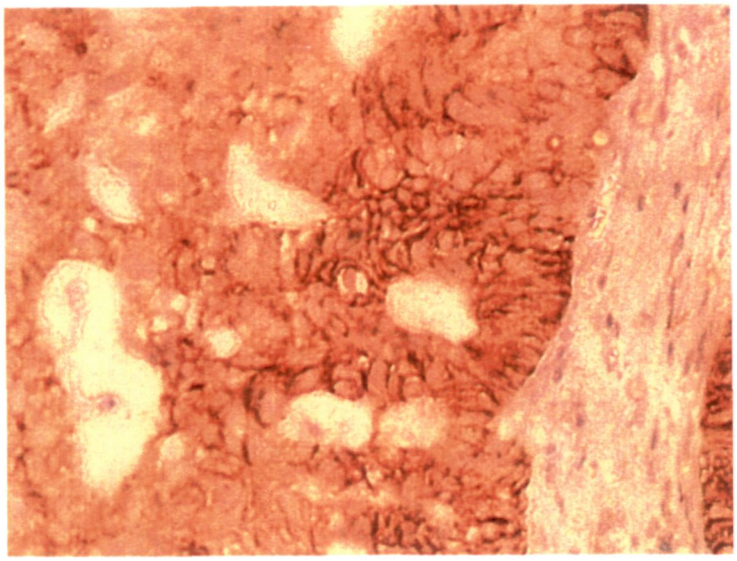


9-6 A

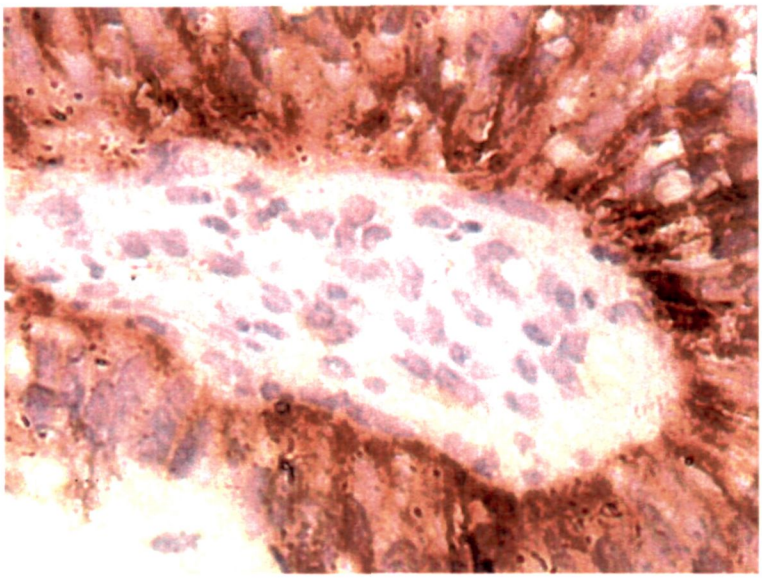


9-6 B

Fig. Slides showing Her-2 neu expression by immunohistochemistry performed on sections of adenocarcinoma of uterine cervix

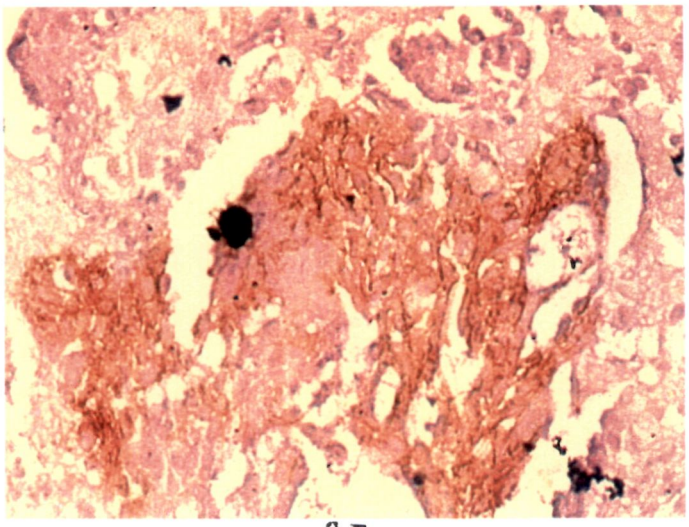


9.6 C



9.6 D

Fig. Slides showing Her-2 neu expression by immunohistochemistry performed on sections of adenocarcinoma of uterine cervix



9.7

Fig Adenosquamous carcinoma

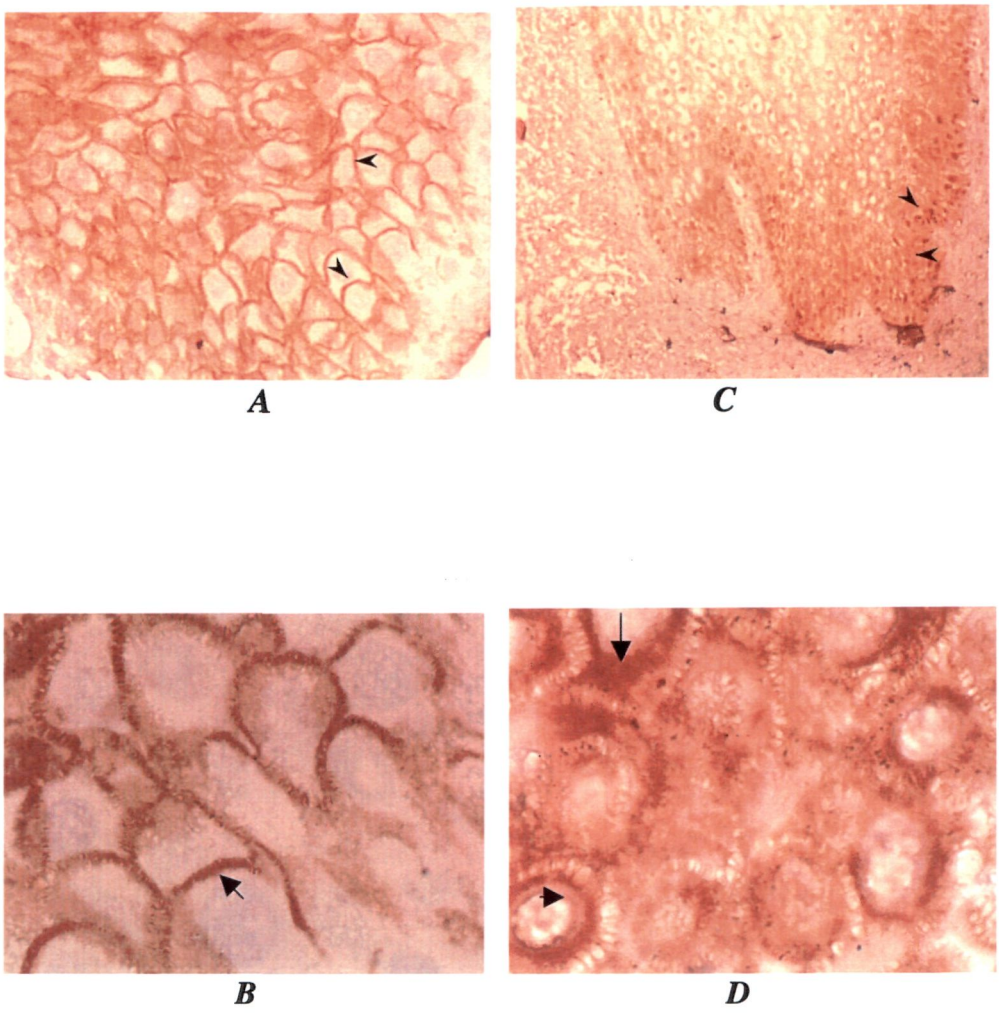


Figure 9.8 Representative photographs, A and B: immunoreactivity of beta catenin in normal cells (control) showing membranous staining whereas C and D: showing predominantly cytoplasmic and nuclear staining with loss of membranous expression of beta catenin (images B and D are under higher magnification).

Discussion

The present study of 50 cervical carcinoma (including 28 squamous cell carcinoma and 22 adenocarcinoma) employing immunohistochemistry showed evidence of frequent *erbB-2* expression. Of the 28 squamous cell carcinoma, 32.1% (9/28), showed *erbB-2* expression, the expression level varied from + (3) to ++ (6). Whereas, in 22 adenocarcinoma 31.8% (7/22) showed *erbB-2* expression and the expression level varied from + (5) to ++ (2). Overexpression of *erbB-2* protein mostly was reported to be associated with erbB-2 gene amplification (Slamon et al., 1987, 1989; Tsuda et al., 1989, 1990; Kihana et al., 1994; Mitra et al., 1994b, 1999). It has also been noted for breast and ovarian. (Slamon et al., 1989; Tsuda et al., 1990). In the present study 7/22 (31.8%) of adenocarcinoma revealed erbB-2 expression. *erb*B-2 (HER-2/neu) oncogene that encodes a 185 kDa receptor tyrosine kinase, which is homologous to human epidermal growth factor receptor (EGFR), have been reported to be overexpressed in adenocarcinomas (Kihana et al, 1994), intraepithelial neoplasia and invasive cancer of the uterine cervix (Pinion et al, 1991; Mitra et al., 1994b; Soh et al., 2002). In our previous study, expression of *erbB-2/ HER-2 neu* was found to be insignificant (8.6%) in cervical dysplasia [unpublished data], suggesting that expression of erbB-2 oncogene is a late event in the process of cervical carcinogenesis. Overexpression of the *HER-2/neu* enhances the malignancy and metastatic potential of

cancer cells and induces the resistance to chemotherapeutic agents provides a scientific basis for the poor clinical outcome associated with HER2/neu overexpressing cancers and overexpression of HER2/neu gene correlates with the relapse and survival rates associated with many human cancers, including breast and ovarian cancers. In a previous study of 22 protooncogenes, Mitra et al (1994b), showed that *erbB-2 (HER-2/neu)* oncogene was frequently amplified in SCC of uterine cervix. We, in the present study, screened the two high-risk HPV types (type 16 and 18), a significant association ($p<0.05$) between HPV type 16 infections and erbB-2 overexpression was observed, as compared to *erbB*-2 negative cases (see Chart 3). 88.8% of the cases showing erbB-2 expression were positive for particular HPV type (type 16). Further, a strong correlation ($p<0.05$) between HPV type 16 infection and erbB-2 overexpression was observed in higher-grade lesions. HPV type 16 was present in 80% of the moderately differentiated squamous cell carcinoma with erbB-2 expression and 25% in erbB-2 negative tumors. Similarly, HPV type 16 was present in all the cases of well-differentiated squamous cell carcinoma with erbB-2 expression as compared to erbB-2 negative tumors (25%).

In the present study, non-cancerous epithelium expressed β-catenin on cell-cell boundaries without exception, whereas, 50% (14/28) of the cancers had reduced beta-catenin expression. 6/13 (46.5%) of the moderately differentiated squamous cell carcinoma and 8/12 (66.6%) of

well differentiated squamous cell carcinoma showed reduced beta-catenin expression, of which, 50% of the moderately and 62.5% of well differentiated tumors were positive for HPV type 16. The reduction in immunoreactivities of the cancer cells might be acquired through malignant transformation. Strong correlation with β-catenin expression and histological grade has been observed in colorectal cancer, however, no such correlation was observed in esophageal or gastric cancer (Takayama et al., 1996). Utsunomiya et al., (2001) also observed overexpression of β-catenin in the cytoplasm and nucleus and reduced expression on cell-cell boundaries in colon cancers. Since it was revealed that β-catenin is involved not only in the cadherin cell adhesion system but also in the growth signal pathway. Beta-catenin binds with the APC tumor suppressor gene product in the cytoplasm, and this complex does not include cadherin (Rubinfeld et al., 1995). The APC gene has been found to be mutated in 80% of colorectal cancers (Miyoshi et al., 1992) and most mutations occurred at beta-catenin binding site. The function of APC as tumor suppressor gene might bind and limit the cytosolic beta-catenin. β-*catenin* in this status binds to Tcf/Lef transcription factors. Soluble beta-catenin plays an important role as a signal transducer that binds and activates the Tcf/Lef transcription factors (Korinek et al., 1997). β-Catenin/Tcf has been widely regarded as the major pathway for carcinogenesis. In association with Tcf, it is translocated to nucleus where it is thought to promote

transcription of genes involved in differentiation and proliferation (Peifer, 1997). Several studies have shown overexpression of β-catenin in human cancers (Takayama et al., 1998; Davies et al., 1998; Yutaka et al., 1999; Huang et al., 1998; Alman et al., 1997; Nhieu et al., 1999). Our results indicate the relationship between beta catenin expression and histological grade. Reduced beta catenin expression was more frequently observed in well-differentiated carcinoma as compared to poorly differentiated squamous cell carcinoma.

Furthermore, in the present study *erbB*-2 expression in tumors with reduced beta-catenin expression was found to be significantly higher 50%(7/14) as compared to preserved beta-catenin expression 14.2% (2/14) ($p<0.05$). Recently, it has been revealed that beta-catenin mediates the interactions of the cadherin catenin complex with the *erb*B-2 gene product and epidermal growth factor receptor (Hoschuetzky et al., 1994; Ochiai et al., 1994; Kanai et al., 1995). Since E-cadherin is suggested to be the major cell adhesion molecule in the mammary gland, in the cytoplasm E-cadherin is linked to beta-catenin and alpha-catenin which mediate the connection of the cytoskeleton, Nagae et al., (2002), in invasive ductal carcinomas using immunohistochemistry, observed, erbB-2 oncoprotein causing disruption of this cell adhesion system through beta-catenin phosphorylation. These results suggest that the E-cadherin-mediated cell adhesion system is frequently lost in invasive ductal-type breast cancers by random loss of E-cadherin/catenins or

erbB-2 overexpression, and that the preservation of this system correlates with well-differentiated morphological features. Ougolkov et al., (2000) in early gastric cancer reported 12 out of 14 cases with erbB-2 overexpression also showing altered beta-catenin expression, suggesting that both molecules are involved in the development of a certain set of differentiated EGCs. Takata et al., (1999) in his study of 31 tumor samples of extramammary Paget's disease reported erbB-2 overexpression but observed no activation of beta-Catenin gene possibly because the pathogenesis of this skin cancer could be different from other common epithelial malignancies.

Therefore it is presumed that along with human papillomavirus infection other genetic changes are involved in the process of cervical carcinogenesis which appear to act either in conjunction with HPV or independently.

General Conclusion

GENERAL CONCLUSION

Cancer of the uterine cervix is the second most common cancer among women worldwide. Epidemiologic studies reveal multiple etiologic factors responsible for the development of cancer, these include infection with certain oncogenic types of human papillomaviruses (HPV), socio-economic factors like, multiparity, multiple sexual partners etc. Extensive viral carcinogenesis studies world over point to human papillomavirus virus as an important etiologic factor in cervical carcinogenesis. Of the many HPV types, types 16 and 18 are documented as high-risk HPV associated with genital neoplasia. Although a cause and effect relationship between HPV and cervical cancer is yet to be proven, over 80% of cervical cancers today are associated with HPV infection. HPV type 16, which is considered to be high-risk type for the pathogenesis of cervical cancer, was present in 42% of cervical cancer (Squamous cell carcinoma), whereas HPV type 6 (LR-HPV) was present in 75% in squamous cell carcinoma (SCC). Co-infection of HPV type 6 and 16 was also present in a considerable number of cases (28%) in squamous cell carcinoma; studies indicate infection of multiple DNA types to be a risk factor for cervical intraepithelial neoplasia (Liaw et al., 1995). E6 of HPV type 6 augments the capability of E7 of HPV type 16 and E7 of type 6 promotes the potential of HPV type 16 in immortalizing normal cell

(Halbert et al., 1992). However, several serologic evidences suggest the interaction of different HPV types. Both HPV types 6 and 16 have antagonistic interference in the respect that HPV type 6 provides immunity against HPV 16 infection These studies suggest that the detection of low-risk HPV types and their co-infection along with HR-HPVs to be important for understanding the pathogenesis of the disease and the biological behavior of HPVs during the process of cervical carcinogenesis.

Further, studies indicate that infection of HPV alone is not sufficient for transforming a normal cell into malignant one, indicating involvement of additional genetic alterations in the progression of cervical cancer, either independently or in conjunction with other etiological factors including HPV infection. Allelic loss and deletion mapping using microsatellite markers and the detection of homozygous deletions represented until now the most powerful method to localize potential tumor suppressor genes. The recurrent genetic alterations can take several forms such as point mutation, viral DNA integration or LOH. Currently, the consistent observation of LOH for markers at a specific location in a series of tumors of a particular histological type is considered to be a marker for loss of function of one or more tumor suppressor genes in the marked genomic region. Frequent LOH at a given locus implies the existence of a candidate tumor suppressor gene that is important in the pathogenesis of the particular cancer under study.

LOH is being detected with increasing frequency in a wide variety of human tumors. Therefore the identification of recurrent chromosomal locations is important for the understanding of the biology of cancer. The genetic alterations in cervical cancer might be either a consequence of biological selection for specific chromosome locations containing integrated HPV DNA or result from other alterations that are recurrently effected in this tumor.

On the other hand mutations in DNA mismatch repair genes result in the accumulation of mutations. The cell with a defect in any of these genes will invariably progress to develop cancer (Kinzler and Vogelstein, 1998). Assessment of microsatellite instability (MSI) has become an important tool in tumor molecular pathology. In the present study we used the Bethesda Conference reference panel of five primers for the first time in cervical cancer. $(A)_n$ tracts located in the intron of *c-kit* oncogene showed maximum (22.9%) instability, followed by the dinucleotide (CA)n repeat loci D2S123 (20.4%). However, BAT-26, in intron 5 of *hMSH2* gene also showed little (9.6%) instability. However, D5S346 showed 3.6% instability, which is significantly low. 13.2% instability was reported at D17S250. Our study supports the results obtained by Dietmaier et al, 1997 in human non-polyposis colorectal cancer (HNPCC), for which the above panel of markers was first recommended. Since, particular repeat loci are prone to instability in different cancers, Dietmaier et al 1997 reported high MSI in the (A)n

tracts located in the intron of c-kit oncogene (BAT-25) confirming the susceptibility of mononucleotide repeats to instability. The present study indicates that a panel of mono and di nucleotide repeat loci that have initially been recommended for HNPCC (Boland et al., 1997), can be used for the assessment of MSI in cervical carcinogenesis which may prove useful from a clinical diagnostic point of view.

In the present study five different polymorphic markers mapped to chromosomal arms 3p and 5p were used to detect LOH. Markers were selected for their reported high LOH rate in other cancers and cervical cancer (Ku et al., 1977; Yokota et al., 1989; Jones and Nakamura, 1992; Kohno et al., 1993; Karlsen et al, 1994; Mitra et al., 1994a; Mullokandov et al, 1996; Larson, 1997; Chu et al., 1998). Of the 83 squamous cell carcinoma, maximum frequency of LOH (42.6%) was observed at D5S208 (5p15.1-15.3) followed by D5S406 (32%) mapped to 5p15.1-15.2. Of the three polymorphic markers mapped to chromosome 3p14.2 (D3S1234, D3S1300 and D3S1313) the most frequently altered loci were D3S1234 (30.2%) within intron 5 of the *FHIT* gene followed by D3S1300 loci (27.6%). Allelotype instability at 3p14.2 suggests inactivation of the *FHIT* gene, which maps to this chromosomal band (Larson et al., 1997; Kohno et al., 1993; Ku et al., 1997 and Chu et al., 1998). Our result confirms a high frequency of allelic imbalances at chromosome arm 3p and 5p indicating the possible existence of candidate tumor suppressor genes involved in cervical

carcinogenesis. Our result also indicates the involvement of FHIT gene in cervical cancer.

LOH and MSI were found to be two independent events occurring during the process of cervical carcinogenesis

Involvement of various oncogenes has been reported in several human cancers. It has recently been reported that *erbB-2* oncogene is overexpressed in adenocarcinomas of uterine cervix (Kihana., et al., 1994) and in CIN and invasive cancer of the cervix (Mitra et al., 1994b; Soh et al., 2002). Oncogene *erb*B-2 (*Her-2/neu)*, which has been reported to be overexpressed in adenocarcinomas of uterine cervix and squamous cell carcinomas of uterine cervix, was studied on 50 cervical cancer cases (including 28 squamous cell carcinoma and 22 adenocarcinoma) employing immunohistochemistry. The results showed evidence of frequent *erbB-2* expression. Overexpression of the *Her-2/neu* enhances the malignancy and metastatic potential of cancer cells and induces the resistance to chemotherapeutic agents. Further a significant association between HPV type 16 infections and *erbB2* overexpression in different histologic types of SCC was observed.

Reduced β-catenin expression at the cell membrane is associated with cancer progression. Overexpression of beta-catenin in the cytoplasm or nucleus is often found in cancers of various organs, including the large intestine (Takayama et al., 1998), endometrium, ovaries (Davies et al., 1998), esophagus (Yutaka Kimura et al., 1999),

thyroid (Huang et al., 1998), soft tissue (Alman et al., 1997) and liver (Nhieu et al.,1999). Therefore the localization (cell membrane, nuclei or cytoplasm) of β-catenin is of considerable importance. Our results show the relationship between beta catenin expression and histological grade. Reduced beta catenin expression was more frequently observed in well-differentiated carcinoma as compared to poorly differentiated squamous cell carcinoma. In order to investigate the cause of increased cytoplasmic staining of *β-catenin* in well differentiated squamous cell carcinoma, it is suggested to analyze the *β-catenin* gene (CTNNB1) using PCR-SSCP analysis. Exon 3 of *β-catenin* gene with nuclear overexpression has already been investigated and was found that mutations in CTNNB1 can increase free cytoplasmic *β-catenin* in colon cancers (Morin et al., 1997). Reduced beta-catenin expression was observed in 46.5% of moderately differentiated squamous cell carcinoma and 66.6% of well differentiated squamous cell carcinoma, of which, 50% of the moderately and 62.5% of well differentiated tumors were positive for HPV type 16. It is likely that accumulation of *β-catenin* in cytoplasm starts in late stage and could pass into the nucleus (nuclear translocation) and interact with transcription factors causing progression to cancer.

Cigarette smoking is a risk factor for cervical neoplasia. Cytochrome P450s that constitute superfamily of enzymes crucial for the oxidative, peroxidative and reductive metabolism of a diverse group

of compounds like endobiotics, xenobiotics that include most of the therapeutic drugs and environmental pollutants (Nelson et al., 1996; Bertz and Granneman, 1997). Nicotine and cotinine have been found to be concentrated in cervical mucus which can be mutagenic (Winkelstein, 1990; Gram et al., 1992; Burger et al., 1993), and DNA from cervical epithelial cells of smokers contains adducts of the type expected from reaction with polycyclic aromatic hydrocarbons and aromatic amines (Simons et al., 1993). This may explain the biological plausibility of the association between smoking and cervical cancer. The expression of CYP enzymes varies between individuals due to genetic and environmental factors and some diseases. Most individuals receiving certain drug like debrisoquine, a beta adrenergic blocking agent prescribed for the treatment of hypertension. excrete large amounts of hydroxylated debrisoquine metabolites in their urine, and they are termed "extensive metabolizers" (EM phenotype) whereas individuals that excrete the drug virtually unchanged are termed "poor metabolizers"(PM phenotype) (Idle et al., 1979). In the present study of 77 normal and 194 cases comprising 61 mild/moderate dysplasia, 48 severe dysplasia and 85 squamous cell carcinoma, a significant influence of CYP2D6 and susceptibility to cervical cancer was observed in women who smoked.

The analysis of the data indicates the involvement of various genetic alterations in the development of cervical cancer. LOH at 3p and

5p was found to be frequent in these cancers. 32.1 percent of cervical cancer cases show high expression of erbB-2 however; higher expression of erbB-2 was seen more frequent in tumors having HPV-16 infection. Reduced expression of beta catenin was found to be more in well differentiated cervical cancers. The data indicates further study on a larger cohort of cervical cancer for a comprehensive view.

Bibliography

Aadrian, J.C., van Den Brule, Chris, J.L.M.M., Victor Bakels., Peter, K.and Walboomers, J.M.M., 1990. Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type specific polymerase chain reaction. *J Clin Microbiol.* **28** (12), 2739-2743.

Aaltonen L.A., Peltomaki P, Leach F.S., Sistonen P., Pylkkanen L., Jukka-Pekka M., Jarvinen H., Powell S.M., Hen J., Hamilton S.R., Petersen G.M., Kinzler K.W., Vogelstein B., de la Chapelle A. 1993. Clues to the pathogenesis of familial colorectal cancer. *Science.* **260.** 810-812.

Aaltonen L.A., Peltomaki P, Mecklin J. P. 1994. Replication errors in benign and malignant tumors from hereditary non polyposis colorectal cancer patients. *Cancer Res.* **54:** 1645-48.

Aareleid T., Pukkala E., Thomson H., Hakama M. 1993. Cervical cancer incidence and mortality trends In Finland and Estonia: a screened *vs* an unscreened population. *Eur. J. Cancer.* **29A.** 745-749.

Al-Kasspooles M., Moore J.H., Orringer M.B., Beer D.G. 1993. Amplification and overexpression of the EGFR and erbB-2 gene in human oesophageal adenocarcinomas. *Int J Cancer.* **54.** 213-219.

Alman B. A., Li C., Pajerski M. E.,Wolfe H. J.1997. Increased beta-catenin protein in somatic APC mutations in sporadic fibromatoses (desmoid tumors). *Am J Pathol.* **151.** 329-334.

Arzimanoglou I. I., Gilbert F., Barber H. R. K. 1998. Microsatellite instability in human solid tumors. *Cancer.* **82.** 1808-1820.

Arzimanoglou I. I., Lallas T., Osborne M. 1996. Microsatellite instability differences between familial and sporadic ovarian cancers. *Carcinogenesis.* **17.** 1799-1804.

Atkin N.B. 1997. ytogenetics of carcinoma of the cervix uteri: a review. *Cancer genet. Cytogenet.* **95.** 33-39.

Ayesh R., Idle J.R., Ritchie J.C., Crothers M.J., Hetzel M.R. 1984. Metabolic oxidation phenotypes as markers for susceptibility to lung cancer. *Nature.* **312.** 169-170.

Baker T.S., Newcomb W.W., Olson N.H., Cowsert L.M., Olson C., Brown J.C. 1991. Structure of bovine and human papillomaviruses: analysis by cryoelectron microscope and three dimensional image reconstruction. *Biophys. J.* **60.** 1445-1456.

Barton S. E., Jenkins D., Cuzik J. 1988. Effect of cigarette smoking on cervical epithelial immunity: a mechanism for neoplastic change. *Lancet.* **2.** 652-654.

Battista P., Palmirotta R., Witullo P. 1997. Microsatellite instability in early gastric cancer. *Int J Cancer.* **10.** 65-70.

Beaudenon S., Kremsdorf D, Croissant O, Jablonska S. et al. 1986. A novel type of human papillomavirus associated with genital neoplasias. *Nature (London).***321.**246-249.

Beaudenon S., Kremsdorf D, Croissant O, Jablonska S. et al. 1986. A novel type of human papillomavirus associated with genital neoplasias. *Nature (London).* **321.**246-249.

Behrens J. 1999. Cadherins and catenins; role in signal transduction and tumor progression. *Cancer Metastasis. rev.***18:** 15-30.

Bell D.A., Taylor J.A., Paulson D.F., Robertson C.N., Mohler J.L., Lucier G.W. 1993. Genetic risk and carcinogen exposure: a common inherited defect of the carcinogen metabolism gene glutathione-S-Transferase M1 (GSTM1) that increases susceptibility to bladder cancer. *J. Natl.Cancer Inst.* **85.** 1159-1164.

Beral V., Hermon C., Munoz N., Devesa S.S. 1994. Cervical cancer. *Cancer Surv.* **19/20.** 265.

Bertz R.J., Granneman G.R. 1997. Use of in vitro and in vivo data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clin Pharmacokinet.* **32(3).** 210-58.

Bishop J.M. 1991. Molecular themes in oncogenesis. *Cell.* **64.** 235-248.

Bishop J.M., Weinberg R.A. eds. 1996. Molecular Oncology. New York: *Scientific American.* Inc.

Boland C.R., Thebodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Digas MA, Fodde R, Ranzani GN and Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: Development of international criteria for the determinaion of microsaellite instability in colorectal cancer. *Cancer Res.* 1998. **58:** 5248-5257.

Bosch F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen, A.M., Peto, J., Schiffman, M.H., Moreno, V., Kurman, R., Shah, K.V., Alihonou, E., Bayo, S., Mockhtar, H.C., Chicreon, S., Daudt, A., Delosrios, E., Ghadirian, P., Kitinya, G.N., Koulibaly, M., Ngelangel, C., Tintore, L.M.P., Riosdalenz, J.L., Sarjadi, A., Schneider, A., Tafur, L., Teyssie, A.R., Rolan, P.A., Torroella, M., Tapia, A.V., Wabings,

H.R., Zatonski, W., Sylla, B., Vizcaino, P., Magnin, D., Kaldor, J., Greer, C., Wheeler, C.,1995. Prevalence of Human Papillomavirus in Cervical Cancer: A worldwide perspective. *J Nat Cancer Institute.* **87**, 796-802.

Boshart M., Gissmann L, Ikenberg H, Kleinheinz A, zur Hausen. 1984. A new type of human papillomavirus DNA, its presence in genital cancer biopsiesand in cell lines derived from cervical cancer. *EMBO J.*. **3.** 1151-1157.

Brauch H, Johnson B, Hovis J, Yano T, Gazdar A, Pettengill OS, Graziano S, Sorenson GD, Poiesz BJ, Minna J. 1987. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. *N Engl J Med.* **317(18).**1109-13.

Brentnall T. A., Chen R., Lee J. G. 1995. Microsatellite instability and K-*ras* mutations associated with pancreatic carcinomas and pancreatitis. *Cancer Res.* **55.** 4264-4267.

Brinton L. A., Schairer C., Haenszel W., Stolley P., Lehman H. F., Levine R. 1986. Cigarette smoking and invasive cervical cancer. *JAMA.* **255.** 3265-3269.

Brinton L.A., Fraument J.F. Jr. 1986b. Epidemiology of uterine cervical cancer. *J. Chron. Dis.* **39.** 1065.

Brodeur G.M., Seeger RC, Schwab M, Varmus HE, Bishop JM. 1984. Amplification of N-*myc* in untreated human neuroblastoma correlates with avanced disease stage.. *Science.* (Washington DC). **224.** 1121-1124.

Broker, T.R., Jin, G., Croom-Rivers, A., Bragg, S.M., Richardson, M., Chow, L.T., Vermund, S.H., Alvarez, R.D., Pappas, P.G., Squires, K.E., Hoesley, C.J., 2001. Viral latency: the papillomavirus model. *Dev. Biol.* **106,** 443–451.

Burger M. P. M., Hollema H., Gouw A.S.H., Pieters W.J.L.M., Quint W.G.V. 1993. Cigarette smoking and human papillomavirus in patients with reported cervical cytological abnormality. *Br Med J.* **306.** 749-752.

Burks R. T., Kessis T. D., Cho K. R. 1994. Microsatellite instability in endometrial carcinmoma. *Oncogene.* **9.** 1163-1166.

Caduff R. F., Johnston C. M., Svoboda-Newman S. M., 1996. Clinical and pathological significance of microsatellite instability in sporadic endometrial carcinoma. *M J Pathol.* **14.** 1671-1678.

Cannizzaro L.A., Durst M., Mendez M.J., Hecht B.K., Hecht F. 1988. Regional hromosome localization and human papillomavirus integration sites near fragile sites, oncogenes, and cancer chromosome break points. *Cancer genet. Cytogenet.* **33.** 93-98.

Caporaso N.E., Tucker M. A., Hoover R.N., Hayes R.B., Pickle L.W., Issaq H.J., Muschik G.M., Green-Gallo L., Buivys D., Aisner S., Resau J.H., Trump B.F., Tollerud D., Weston A., Harris C. C. 1990. Lung cancer and the debrisoquine metabolic phenotype. *J Natl. Cancer Inst.* **82.** 1264-1272.

Chaouki N., Bosch FX, Munoz N, Meijer CJLM, Gueddar BE, Ghazi AE, Deacon J, Castellsagu JX, Walboomers JMM. 1998. The viral origin of cervical in Rabat. Morocco. *Int. J. Cancer.* **75;** 546-54.

Chong J., Fukayama M., Hayashi Y. 1994. Microsatellite instability in the progression of gastric carcinoma. *Cancer Res.* **54.** 4595-4597.

Christofori G., Semb H. 1999. The role of the cell-adhesion molecule E-cadherin as a tumour suppressor gene. *Trends Biochem Sci.* **24.** 73-75.

Chu T.Y., Shen CY, Lee HS, Liu HS. 1999. Monoclonality and surface lesion-specific microsatellite alterations in premalignant and malignant neoplasia of uterine cervix: a local field effect of genetic instability and clonal evolution. *Genes Chromosomes Cancer.* **24.** 127-134.

Chu T.Y., Shen CY, Perng CL, Yu MS, Liu HS. 1998. HPV-associated cervical cancers show frequent allelic loss at 3p14 but no apparent abberation of FHIT mRNA. *Int J Cancer.* **75.** 199-204.

Chung G. T., Huang D.P., Lo K.W., Chan M.K., Wong W. 1992. Genetic lesion in the carcinogenesis of cervical cancer. *Anticancer Res.* **12.** 190.

Chung T. K. H., Ip T. Y., Hampton G. M., Wang V. W., Cheung T. H., Wong Y. F. 2001. Microsatellite instability in cervical carcinoma. *Eur J Obstet Gynecol Reprod Biol.* **94**. 121-124.

Clavel C., Masure M, Putaud I, et al. 1998. Hybrid capture II, a sensitive test for human papillomavirus detection. Comparision with Hybrid capture I and PCR results in cervical lesions. *J. Clin. Pathol.* **51**.: 737-740.

Clifford G.M., Smith J.S., Plummer M., Munoz N., Franceschi S. 2003. Human papillomavirus types in invasive cervical cancer worldwide: meta analysis. *Br J. Cancer.* **88.** 63-73.

Cole S.T. , Danos O. 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of

papillomaviruses and repeated structure of the E6 and E7 gene products. *J Mol Biol.* **193.** 599-608.

Cole S.T., Streeck RE. 1986. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. *J Virol.* **58.** 991-995.

Coleman M., Esteve J., Damiecki P., Arslan A., Renard H. 1993. Times trends in cancer incidence and mortality. *(IARC Scientific Publications. No. 121). Lyon IARC.*

Cook G.A.., Draper G.J.,1984 Trends in cervical cancer and carcinoma in situ in Great Britain. *Br. J. Cancer*; **50**, 367-75.

Coombs L.M., Pigott DA, Sweeny E, Proctor AJ, Eydmann ME, Parkinson C, Knowles MA. 1991. Amplification and overexpression of *c-erbB-2* in transitional cell carcinoma of the urinary bladder. *Br J Cancer.* **63.** 601-608.

Cullen A.P., Reid R., champion M., Lurinez A.T. 1991. Analysis of the physical state of diferent Human Papillomavirus DNAs in intraepithelial and invasive cervical neoplasms. *Journal of Virology.* **65**: 606-612.

Cullen A.P., Reid R., champion M., Lurinez A.T. 1991. Analysis of the physical state of diferent Human Papillomavirus DNAs in intraepithelial and invasive cervical neoplasms. *Journal of Virology.* **65**: 606-612.

Cuzick J., Boyle P. 1988. Trends in cervix cancer mortality. *Cancer Surv.***7.** 417-439.

Danos O., Katinka M, Yaniv M. 1982.Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among *Papoviridae. EMBO J.* **1.** 231-236.

Danos O., Katinka M, Yaniv M. 1982.Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among *Papoviridae. EMBO J.* **1.** 231-236.

Das B.C., Gopalkrishna V., Hedau S. Katyar S. 2000. Cancer of the uterine cervix and human papillomavirus infection. *Current Science.* **78;** 52-63.

Das B.C., Sharma J.K., Gopalkrishna V., Das D.K., Singh V., Gismann L., zur Hausen H., Luthra U.K. 1992. *Journal of Medical Virology.* **36:** 239-245.

Davies B. R., Worsley S. D., Ponder B. A.1998 Expression of E-cadherin, alpha-catenin and beta-catenin in normal ovarian surface epithelium and epithelial ovarian cancers. *Histopathology.* **32.** 69-80.

de Roda Husman, A. M., Walboomers, J. M. M., Van Den Brule, A. J. C., Meijer, C. J. L. M., Snijders, P. J. F. 1995. The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J. Gen. Virol. 76, 1057-1062.

Devilee P, van den Brock M., Dupers-Dijkshoorn N., Khan P., Pearson P., Cornelisse C. 1989. At least four frequent chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. *Genomics.* **5.** 554-560.

Dietmaier W., Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. 1997. Diagnostic microsatellite instability : definition and correlation with mismatch repair protein expression. *Cancer Res.*. **57;** 4749-4756.

Dos Santos N. R., Seruca R., Constancia N. 1996. Microsatellite instability at multiple loci in gastric carcinoma: clinical pathological implications and prognosis. *Gastroenterology* **110.** 38-44.

Dracopoli N.C., Fogh J. 1983. Loss of heterozygosity in cultured human tumor cell lines. *J. Natl. Cancer Instt.* **70.** 83-87.

Duggan B. D., Felix J. C., Muderspach L. I. 1994. Microsatellite instability in sporadic endometrial carcinoma. *J Natl Cancer Inst.* **86.** 1216-1221.

Dürst M., Gissmann H, Ikenberg H, H zur Hausen. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl. Acad. Sci.* USA. **80.** 3812-3815.

Dürst M., Gissmann H, Ikenberg H, H zur Hausen. 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl. Acad. Sci.* USA. **80.** 3812-3815.

El-Naggar A.K. 1995. Sequential loss of heterozygosity at microsatellite motifs in pre-invasive and invasive head and neck squamous carcinoma. *Cancer Res.* **55.** 2556-2559.

Evan G. I. Vousden K. H. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature.* **411.** 342-348.

Favre M., Croissant O, Orth G. 1989a. Human papillomavirus type 29 (HPV 29), an HPV type cross hybridization with HPV 2 and with HPV 3 related types. *J Virol.* **63.** 4906.

Favre M., Obalek S, Jablonska S, Orth G. 1989b. Human papillomavirus type 28 (HPV-28), an HPV-3 related type associated with skin warts. *J. Virol.*. **63.** 4905.

Favre M., Obalek S, Jablonska S, Orth G. 1989c. Human papillomavirus type 49, a type isolated from flat warts of renal transplant patients. *J Virol.* **63.** 4909.

Favre M., Obalek S, Jablonska S, Orth G. 1989d. Human papillomavirus (HPV) type 50, a type associated with epidermodysplasia verruciformis (EV) and only weakly related to other EV-specific HPVs. *J Virol.* **63.** 4910.

Ferenczy A., Franco E. L. 2002. Persistent human papillomavirus infection and cervical neoplasia. *Lancet Oncol.* **3.** 11-16.

Fey M.F., Hesketh C, Wainscoat JS, Gendler S, Thein SL. 1989. Clonal allelic loss in gastrointestinal cancers. *Br J cancer.* **59.** 750-754.

Franco E. L., Rohan T. E., Villa L. L.1999. Epidemiologic evidence and human papillomavirus infection as a necessary cause of cervical cancer. *J. Natl. Cancer Inst.* **91,** 506-511.

Franco E.L., Villa L.L., Ruiz A., Costa M.C., 1995. Transmission of cervical human papillomavirus infection by sexual activity: differences between low and high-risk types. *J. Infect. Dis.* **172.** 756-763.

Freeman M.R., Washeeka R, Chung L W. 1989. Aberrant expression of epidermal growth factor receptor and Her-2 (erb-2) mRNAs in human renal cancers. *Cancer Research.***49**: 6221-6225.

Fujita M., Inomoto T., Yoshino K. 1995. Microsatellite instability and alterations in the hMSH2 gene in human ovarian cancer. *Int J Cancer (Pred Oncol).***64.** 361-366.

Fukushima M., Yamakawa Y., Shimano S., Hashimoto M., Sawada Y., Fuginaga K. 1990. The physical state of human papillomavirus 16 DNA in cervical carcinoma and cervical intraepithelial neoplasia. *Cancer.* **66.** 2155-2161.

Gao X., Wu N., Grignon DD. 1994. High frequency of mutator phenotype in human prostatic adenocarcinoma. *Oncogene.* **9.** 2999-3003.

Gissmann L, de Villiers, E. M., zur Hausen H. (1982). Analysis of human genital warts (condyloma accuminata) and other genital tumors for human papillomavirus type 6 DNA. *Int. J. Cancer.* **29.** 143-146.

Gissmann L., Pfister H, H zur Hausen. 1977. Human papillomavirus (HPV): chracterization of four different isolates. *Virology.* 569-580.

Gollin S., Ishwad C., Reddy J., Karen R., Appel B., Law J., Johnson J., Myers E., Ferrel R. 1994. Loss of heterozygosity and genomic instability in oral cancer (Abs). *Proc Am Assoc Cancer Res.***35. 117.**

Gonzalez F. J. Nebert D. W. 1990. Evolution of the p450 gene super family: Animal plant "warfare," molecular drive, and human genetic differences in drug oxidation. *Trends Genet.* **6.** 182-186.

Gonzalez Z.M., Ruppert J M, Tokino K, Tsai Y C, Spruck C H III, Miyao N, Nichols P W, Hermann G G, Horn T, Steven K, Summer Hayes I C, Sidransky D, Jones P A. 1993. Microsatellite instability in bladder cancer. *Cancer Research.* **53**: 5620-5623.

Gopalkrishna V., Francis A, Sharma JK, Das BC. 1992. A simple and rapid method of high quantity DNA isolation from cervical scrapes for

detection of human papilloamvirus infection. *J Virol Methods.* **36.** 63-72.

Gough A. C., Miles J. S., Spur N. K., Moss J. E., Gaedigk A., Eichelbaum M., Wolf C. R. 1990. Identification of primary gene defect at the cytochrome p450 CYP2D6 locus. *Nature.* **347.** 773-776.

Graham S.E., Peterson J.A. 1999. How similar are P450s and what can their differences teach us? *Arch Biochem Biophys* **369**: 24-29.

Gram I. T., Austin H., Stalsburg H. 1992. Cigarette smoking and the incidence of cervical intraepithelial neoplasia grade III, and cancer of cervix uteri.*Am J Epidemiol.* 135. 341-346.

Greenspan D.L., Connolly D.C., Wu R., Lei R.Y., Vogelstein J.T.C., Kim Y., Mok J.E. , Munoz N., Bosch F.X., Shah K., Cho K.R. 1997. Loss of FHIT expression in cervical carcinoma cell lines and primary tumors. *Cancer Res.* **57.** 4692-4698.

Guengrich F. P. 1990. Enzymatic oxidation of xenobiotics chemicals. *CRC Crit. Rev. Biochem. Mol. Biol.* **25.** 97-153.

Guo Z., Wilander E., Sallstrom J., Ponten J. 1998. Deletion of chromosome 3p is an early event in malignant progression of cervical cancer. *Anticancer Res.* **18.** 707-712.

Hakama M., Magnus K., Pettersson F. 1991. Effect of organized screening on the risk of cervical cancer in the Nordic countries. In: Miller A.B. Chamberlain J. Day N.E., Hakama M., Prorok P.C. eds. *Cancer Screening. Cambridge. Cambridge University Press* pp 152-162.

Halbert C.L., Demers GW, Galloway DA. 1992. The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells.*J. Virol.* **66:**2125-2134.

Han H. J. Yanagisawa A., Kato Y. 1993. Genetic instability in pancreatic and poorly differentiated type of gastric cancer. *Cancer Res.* **53.** 5087-5089.

Harris R. W. C., Brinton L. A., Cowdell R. H. et al. 1980. Characterstics of women with dysplasia or carcinoma in situ of the cervix uteri. *Br J Cancer.* **42.** 359-369.

Harwood, C.A., Spink, P.J., Surentheron, T., Leigh, I.M., De Villiers, E.M., McGregor, J.M., Proby, C.M., Breuer, J., 1999. Degenerate and nested PCR: a highly sensitive method for detection of human papillomavirus infection in cutaneous warts. *J. Clin. Microbiol.* **37** (11), 3545–3555.

Hayashi S., Tanimoto K, Hajiro-Nakanishi K, Kurosomi M, Higashi Y, Imai K, Suga K, Nagachi K. 1997. Abnormal FHIT transcript in human breast carcinomas: a clinicopathological and epidemiological analysis of 61 Japanese cases. *Cancer Research.* **57.** 1981-1985.

Hayden J. D., Cawkwell L., Sue-Ling H. 1997. Assessment of microsatellite alterations in young patients with gastric adenocarcinoma. *Cancer.* **79.** 684-687.

He T.C., Sparks AB, Rago C, et al. 1998. Identification of *c-myc* as a target of the APC pathway. *Science.* **281.** 1509-1512.

Heck D.V., Yec C, Howley PM, Műnger K, 1992.*Proc. Natl. Acad. Aci. USA.* **89.** 4442-4446.

Heilmann C.A., Law M-F, Israel MA, Howley PM. 1980. Cloning of human papillomavirus genomic DNAs and analysis of hoologous polynucleotide sequences. *J Virol.* **36.** 395-407.

Helland A, Kraggerud SM, Kristensen GB, Holm R, Abeler VM, Huebner K 2000. Primary cervical carcinomas show 2 common regions of deletion at 3P, 1 within the FHIT gene: evaluation of allelic imbalance at FHIT, RB1 and TP53 in relation to survival. *Int J CanceR.* **88.** 217-22

Helland A., Kraggerud S.M., Kristensen G.B., Holm R., Abeler V.M., Huebner K., Borresen-Dale A.L., Lothe R.A. 2000. *Int J Cancer.* **88.** 217-222.

Hellberg D. 1993. Behavior of cervical intraepithelial neoplasia (CIN) associated with various human papillomavirus (HPV) types. *Arch Gynecol Obstet.* **252.** 119-128.

Hildesheim A., Schiffman MH, Gravitt PE, Glass AG, Grer CE, Zhang T, Scott DR, Rush BB, Lawler P, Sherman ME, Kurman RJ and Manos MM. 1994. *J.Infect. Dis.* **169.** 235-240.

Hinck L., Nathke IS, Papkoff J, Nelson WJ. 1994. Dynamics of cadherin catenin complex formation: novel protein interactions and pathways of complex assembly. *J Cell Biolk.* **125.** 1327-1340.

Hoang J.M., Cottu PH, Thuille B et al. 1997. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Res.* **57;** 300-303.

Hoschuetzky H., Aberle H, Kemler R. 1994. β-catenin mediates the interaction of thecadherin catenin complexwith epidermal growth factor receptors. *J Cell Biol.* **127.** 1375-1380.

Hou Y., Wang M, Liu W. 1996. Study of the amplification and expression of *c-erbB2* oncogene in epithelial ovarian tumours. *Chung Hua Chung LiuTsa Chih.* **18(6);** 426-428.

Houldsworth J., Cordon-Cardo C, Ladanyi M, Kelse DP and Chaganti RSK. 1990. Gene amplification in gastric and oesophageal adenocarcinomas. *Cancer Res.* **50.** 6417-6422.

Huang S. H., Wu J. C., Chang K. J., Liaw K. Y. 1998. Distribution of cadherin catenin complex in normal human thyroid carcinoma cell-line. *J Cell Biol.* **70.** 330-337.

Huang Y. et al. 1992. Loss of heterozygosity involves multiple tumour suppressor genes in human oesophageal cancer. *Cancer Research.* **52:** 6525-6530.

Huettner P.C., Gerhard DS, Li L, Gersell DJ, Dunnigan K, Kamarasove T, Rader JS. 1998. Loss of heterozygosity in clinical stage IB cervical carcinoma: relationship with clinical and histopathologic features. *Hum Pathol.* **29.**364-370.

Huibregste J.M., Scheffner M. seminars Virology. 1993.**5;** 357-367.

Huiping C., Kristjansdottir S., Jonasson J.G., Magnusson J., Egilsson V., Ingvarsson S. 2001. Alterations of E-cadherin and beta-catenin in gastric cancer. *BMC Cancer.*

IARC Scientific Publication No. **143,** 1997.

IARC Working Group. 1995. Human papillomaviruses. *IARC Monograph on the evaluation of carcinogenic risk to humans.* Vol. 64. Lyon, France: International Agency for Research on Cancer.

Idle J. R., Armstrong M., Boddy A. V., Boustead C., Cholerton S., Cooper J., Daly A. K., Ellis J., Gregory W., Hadidi H., Hofer C., **Holt J.,**

Lethart J. McCracken N., Monkman S.C., Painter J. E., Taber H., Walker D., Yule M. 1992. The pharmacogenetics of chemical carcinogenesis. *Pharmacogenetiocs.* **2.** 246-258.

Idle J.R., Smith R.L. 1979. Polymorphisms of oxidation at carbon centers of drugs and their clinical significance. *Drug Metab. Rev.* **9.** 301-317.

Ikeda S., Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A. 1998. Axin, a negative regulator for Wnt signalling pathway, forms a complex with GSK-3beta and beta-catenin and promotesGSK-3beta-dependent phosphorylation of beta-catenin. *EMBO J.* **17.** 1371-1384.

Ionov Y., Peinado M A, Malkhosyan S, Shibata D, Perucho M. 1993. Ubiquitious somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature.* (Lond.), **363**: 55-561.

Ionov Y., Peinado S, Malkhosyan et al. 1993. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colorectal carcinogenesis. *Nature.* **363.** 558-561.

Izumoto S., Arita N., Ohnishi T. 1997. Microsatellite instability and mutated type II transforming growth factor-β receptor gene in gliomas. *Cancer Lett.* **112.** 251-256.

Jones M.H., Nakamura Y. 1992. Deletion mapping of chromosome 3p in female genital tract malignancies using microsatellite polymorphism. *Oncogene.* **7.** 1631-1634.

Jussawala D.J, Deshpande V.A., Standfast S.J. 1971. Assessment of risk pattern in cancer of the cervix. A comparison between greater Bombay and Western countries. *Int. J. Cancer.* **7.** 259.

Kanai Y., Ochiai A., Shibata t., Oyama t., Ushijima S., Akimoto S., Hirohashi S. 1995. c-erbB-2 gene product directly associates with β-catenin and plakoglobin. *Biochem. Biophys. Res. Commun.* **208.** 1067-1072.

Karlsen F., Rabbitts PH, Sundresan V, Hagmar B. 1994. PCR-RFLP studies on 3p in formaldehyde-fixed paraffin-embedded cervical cancer tissue. *Int J Cancer.* **58.** 787-792.

Katabuchi H., van Rees B., Lambers A. R. 1995. Mutations in DNA mismatch repair genes are not responsible for microsatellite instability in most sporadic endometrial carcoinomas.. *Cancer Res.* **55.** 5556-5560.

Keller G., Rotter M., Vogelsang H. 1995. Microsatellite instability in adenocarcinoma of the upper gastrointestinal tract. Relation to clinicopathological data and family history. *M J Pathpol.* **147.** 593-600.

Kern S.E.,1989. Allelic loss in colorectal carcinoma. *Journal of American Medical Association.*; **261:** 207-211.

Kersaemaeckers A.M.F., Hermans J, Fleuren G J, van de Vijver M J. 1998a; Loss of heterozygosity for defined regions on chromosome 3, 11 and 17 in carcinoma of the uterine cervix. *British J. Cancer.* **77**: 192-200.

Kersaemaekers A.M.F., Kenter GG, Hermans J, Fleuren GJ, van de Vijver MJ. 1998b. Allelic loss and prognosis in carcinoma of the uterine cervix. *Int J Cancer..* **79.** 411-417.

Khosla S., Patel VM, Hay ID, Schaid DJ, Grant CS, van Heeren JUA, Thibodeau S N. 1991. Loss of heterozygosity suggests multiple genetic alterations in phenochromocytomas and medullary thyroid carcinomas. *J Clin Invest..* **87.** 1691-1699.

Kihana T., Tsuda H, Teshima S, Kiyoki N, Tsugane S, Sonada T,Matsura S, Hirohashi S. 1994; Prognostic significance of the overexpression of *c-erbB-2* protein in adenocarcinoma of the uterine cervix. *Cancer.* **73**: 148-153.

Kihara M., Kihara M., Noda K. 1994. Lung cancer risk of GSTM1 null genotype is dependent on the extent of tobacco smoke exposure. *Carcinogenesis.***15.** 415-418.

Killary A. M., Wolf M.E., Giambernardi T.A., Naylor S.L. 1992. Definition of tumor suppressor locus within human chromosome 3p21-p22. *Proc. Natl Acad. Sci. USA*. **89.** 10877-1081.

Kimura M., Abe T., Sunamura M. 1996.Detailed deletion mapping on chromosome arm 12q un human pancreatic adenocarcinoma: identification of a 1cM region of common allelic loss. *Genes Chromosom Cancer.* **17.** 88-93.

Kimura Y.M., Shiozaki H., Doki Y., Oka H., Inoue M., Yamamoto M., Tamura S., Shibamoto S., Ito., F., Monden M. 1999. Cytoplasmic beta catenin in esophageal cancers. *In.t J. Cancer.* **84**. 174-178.

King B.L., Carcangiu M. L., Carter D. 1995. Microsatellite instability in ovarian neoplasms. *Br J Cancer.* **72.** 376-382.

Kinzler K. W and Vogelstein. 1998. Landscaping the cancer terrain. *Science.* **280.** 1036-1037.

Klingenberg, M. 1958. Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* **75.** 376-386,

Klug A., Finch J.T. 1965. Structure of viruses of papilloma-polyoma type 1. Human papillomavirus.*J. Mol. Biol.***11.** 403-423.

Knudson A.G. 1971. Mutation and cancer: Statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA.* **68.** 820-823.

Knudson A.G. 1985. Hereditary cancer, oncogenes and antioncogenes. *Cancer Res..* **45;** 1437-1443.

Kobayashi K., Matsushima M., Sumiko K. 1996. Mutational analysis of mismatch repair genes, *hMLH1* and *hMSH2*, inn sporadic endometrial carcinomas with microsatellite instability. *Jpn J Cancer Res.* **87.** 141-1454.

Koch A., Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D, Pietsch T. 1999. Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the beta-catenin gene. *Cancer Res.* **59.** 269-273.

Kohno T., Takayama H, Hamaguchi M, Takano H, Yamaguchi N, Tsuda H, Hirohashi S, Vissing H, Shimizu M, Oshimura M, Yokota J. 1993. Deletion mapping of chromosome 3p in human uterine cervical cancer. *Oncogene..* **8.** 1825-1832.

Kok K., Naylor S. L., Buys C. H. 1997.Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv Cancer Res.* **71.** 27-92.

Kok K., Osinga J., Carrit B., Davis M.B., van der Hout A.H., van der Veen A.Y., Landsvater R.M., Leij L.F.M.H., Berendsen H.H., Postmus P.E., Poppema S., Buys C.H.C.M. 1987. Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature.* **330.** 578-581.

Kondo Y., Kanai Y, Sakamoto M, Genda T, Mizokami M, Ueda R, Hiroshi S. 1999. Beta-catenin accumulation and mutation of exon 3 of the beta-catenin gene in hepatocellular carcinoma. *Jpn. J. Cancer Res.*. **90:** 1301-1309.

Korinek V., Barker N, Morn PJ, Van Wichen D, Deweger R, Kinzler KW, Vogelstein B, Clevers H. 1997. Constitutive transcriptional activation by a βcatenin-Tcf complex in APC-/-carcinoma. *Science*..**275.**1784-1787.

Koss L. G. 1963. Some histological aspects of behavior of epidermoid carcinoma in situ and related lesions of the uterine cervix. *Cancer.* **16.** 1160-1211.

Kovacs G., Erlandsson R, Boldog F, Ingvarsson S, Muller-Brechlin R, klein G, Sumegi J. 1988. Consistent chromosome 3p deletion and loss of heterozygosity in renal cell carcinoma. *Proc. Natl. Acad Sci. USA*.. **85.** 1572-1575.

Kremsdorf D., Favre M, Jablonska S, Obalek S, Rueda LA, Lutzner MA, lanchet-Brdon C, van Voorst Vader PC, Orth G. .1984. Molecular cloning and characterization of the genomes of nine newly recognized human papillomavirus types associated with epidermodysplasia verruciformis. *J. Virol.* **52.** 1013-1018.

Kremsdorf D., Favre M, Jablonska S, Obalek S, Rueda LA, Lutzner MA, lanchet-Brdon C, van Voorst Vader PC, Orth G. .1984. Molecular cloning and characterization of the genomes of nine newly reognized human papillomavirus types associated with epidermodysplasia verruciformis. *J Virol.* **52.** 1013-1018.

Kremsdorf D., Jablonska S, Favre M, Orth G. 1983. Human papillomaviruses associated with epidermodysplasia verruciformis II.

Molecular cloning and biochemical characterization of human papillomavirus 3a, 8, 10 and 12 genomes. *J. Virol.*. **48.** 340-351.

Kristiansen E., Genkins A., Holm R. 1994. Co-existence of episomal and integrated HPV 16 DNA in squamous cell carcinoma of the cervix. *J. Clin. Pathol.* **47.** 253-256.

Ku W.H., Liu IL, Yen MS, Chang Chien CC, Yue CT, Ma YY, Chang SF, Ng HT, Wu CW, Shen CY. 1997. Genomic deletion and p53 inactivation in cervical carcinoma. *Int J Cancer.*. **72 (2).** 270-276.

Larson A.A., Liao SY, Stanbridge EJ, Cavenee WK, Hampton GM. 1997. Genetic alterations accumulate during cervical tumorigenesis and indicate a common origin for multifocal lesions. *Cancer Res.*.**57**; 4171-4176.

Lazo P. A. 1999. The molecular genetics of cervical carcinoma. *Br J Cancer.* **80 (12).** 2008-2018.

Lechner M.S., Mack DH, Finicle AB, Crook T, Vousden KH, Laimins LA. *EMBO J.* 1992. **11.** 3045-3052.

Lee J.H., Kavanagh J.J., Wildrick DM, Wharton JT, Blick M. 1990. Frequent loss of heterozygosity on chromosome 6q, 11 and 17 in human ovarian carcinomas. *Cancer res.* **50.** 2724-2728.

Lehn H., Villa L.L., Marziona F., Hilgarth.M., Hillemans H.G., Sauer G. 1988. Physical state and biological activity of human papillomavirus genomes in precancerous lesions of the female genital tract. *J. gen. Virol.* **69.** 187-196.

Lengauer C., KinzlerK. W., Vogelstein B. 1998.Genetic instabilities in human cancers. *Nature.* **396.** 643-649.

Lerman M., Minna J., Sekido Y., Bader S., Burbec D., Fong K., Forgacs E., Gao B., Garner H., Gazdar A. F., Girard L., Kamibayashi C. 2000. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. *Cancer Res.* **60.** 6116-6133.

Liaw K.L., Hsingh A.W., Chen C.J., Schiffman M.H., Zhang T.Y., Hsieh C.Y., Greer C.E., You S.L., Huang T.W., Wu T.C., O'Leary T.J., Seidman .L.,lot W.J., Meinert C.L., Manos M.M. 1995. Human papillomavirus and cervical neoplasia: a case control study in Taiwan. *Int. J. Cancer.* **62.** 565-571.

Lim P. C., Tester D., Cliby W. 1996. Absence of mutations n DNA mismatch repair genes in sporadic endometrial tumors with microsatellite instability. *Clin Cancer Res.* **2.** 1907-1911.

Lin W.M., Eugenia A., Michalopulos N.D., Pui C., Cheng W.R., Ashfaq R., Coleman R.L., Muller C.Y. 2000. Allelic loss and microsatellite alterations of chromosome 3p14.2 are more frequent in recurrent cervical dysplasias. *Clin. Cancer Res.* **6.** 1410-1414.

Loeb L. A., (1991). Mutator phenotype may be required for multistep carcinogenesis. *Cancer Res.* **51.** 3073-3079.

Lorincz A.T., Quinn AP, Goldsborough MD, Schmidt BJ, Temple GF. 1989. Cloning and partial DNA sequencing of two new human papillomavirus types associated with condylomas and low-grade cervical neoplasia. *J Virol.*. **63.** 2829-2832.

Lorincz A.T., Quinn AP, Lancaster WD, temple GF. A new type of human papillomavirus associated with cancer of the uterine cervix. *Virology.* 1987. **159.** 187-190.

Lothe R., Fossa S., Stenwig A., Nakamura Y., White R., Borrensen A., Borgger A. 1989. Loss of 3p and 11p alleles is associated with testicular cancer tumors. *Genomics.* **5.** 134-138.

Lui B., Parsons R. E., Hamilton S. R. 1994. *hMSH2* mutations in hereditary non polyposis colorectal cancer kindreds. *Cancer Res.* **54.** 4590-4594.

Luthra U. K., Prabhakar AK, Seth P, Agarwal SS, Murthy NS, Bhatnagar P, Sharma BK. 1987. Natural history of precancerous and early cancerous lesions of the uterine cervix. *Acta Cytologica.* **31.** 226-234.

Maiti S., Alam R, Amos CI and Huff V. Frequent association of beta-catenin and WTI mutations in Wilms tumor. *Cancer Res.* 2000. **60:** 6288-6292.

Manos M.M., Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinski SM. 1989. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells.***7.** 209-214.

Mao L. 1996. Frequent microsatellite alterations at chromosome 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. *Nature Med* .**2:** 682-685.

Marshal C.J. Tumour suppressor genes. *Cell. I.* 1991; **64**: 313-326.

McWilliams J.E., Cohen J.I., Henner W.D.1996. Glutathione-S-Transferase M1 and T1 deficiencies and head and neck cancer risk. *Proc Am. Assoc. Cancer Res.* **37.** 107.

Meltzer S.J., Yin J., Manin B., Rhyu M.G., Cottrell J., Hudson E., Redd J.L. Krasna M.J., Abraham J.M., Reid B.J. 1994. Microsatellite

instability occurs frequently and in both diploid and aneuploid cell populations of Barrett's associated esophageal adenocarcinomas. *Cancer Res.* **54.** 3379-3382.

Meltzer S.J., Ahnen DJ, battifora H, Yokota J, Cline MJ. 1987. Protooncogene abnormalities in colon cancers and adenomatous polyps. *Gastroenterology..* **92.** 1174-1180.

Mertens F., Johansson B., Hoglund M., Mitelman F. 1997. Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. *Cancer Res.* **57.** 2765-2780.

Meyer U. A., Zanger U. M., Grant D., Blum M., 1990. Genetic polymorphisms of drug metabolism. *Adv Drug Res.* **19.** 197-241.

Miller J.R., Hocking AM, Brown JD, Moon RT. 1999. Wnt/beta-catenin and Wnt/Ca2+ pathways. *Oncogene.* **18.** 7860-7872.

Mironov N. M., Aguelon M. A., Potapova G. I. 1994. Alteration of $(CA)_n$ DNA repeats and tumor suppressor genes in human gastric cancer. *Cancer Res.* **54.** 41-44.

Mitra A.B. 1999. Genetic Alterations and human papillomavirus infection in cervical cancer : Loss of heterozygosity sites at 3p and 5p are important genetic events. *International Journal of Cancer.* **82**: 322-324.

Mitra A.B., Murty V V V S, Pratap M, Sodhani P, Chaganti R S K , 1994b. erbB2 (Her-2/nue) oncogene is frequently amplified in squamous cell carcinoma of the uterine cervix. *Cancer Research.* **54**: 637-639.

Mitra A.B., Murty V V V S., Singh V, Li R G, Pratap M, Sodhani P, Luthra U K, Chaganti R S K. May, 1995 . Genetic Alteraions at 5p15 : A Potential marker for progression of precancerous lesions of the uterine cervix. *Journal of National Cancer Institute.* **87:** 742-745.

Mitra A.B., Murty V.V.V.S., Rong G.I., Pratap M., Luthra U.K., Chaganti R, S.K. 1994a. Allelotype analysis of cervical carcinoma. *Cancer Res.* **54:** 4481-4487.

Miyoshi Y., Iwao K., Nawa G., Yoshikawa H., Ochi T., Nakamura Y. 1998. Frequent mutations in the beta-catenin gene in desmoid tumours from patients without familial adenomatous polyposis. *Oncol. Res.* **10.** 591-594.

Miyoshi Y., Nagase H., Ando H., Horii A., Ichii S., Nakatsuru S., Aoki T., Miki Y., Mori T., Nakamura Y. 1992. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Hum. Mol. Genet.* **1.** 229-233.

Morin P. J. 1999. Beta catenin signalling and cancer. *Bioessays.* **21.** 1021-1030.

Morin P. J., Sparks A.B., Korinek V., Barker N, Clevers H., Vogelstein B., Kinzler K.W. 1997. Activation of beta-catenin-Tcf signalling in colon cancers by mutations in beta-catenin or APC. *Science.* **275.** 1787-1790.

Muller M.G., Kelly M, Fiedler M, Gissmann L. 1987. Human papillomavirus type 48. *J Virology.***63.** 4907-4908.

Mullokandov M.R., Kholodilov N G, Atkin N B, Burk R D, Johnson A B, Klinger H P. 1996. Genomic alterations in cervical carcinoma: Losses

of chromosome heterozygosity and human papillomavirus tumour status. *Cancer Research.* **56**: 197-205.

Munemitsu S., Albert I, Souza B, Rubinfeld B, Polakis P. 1995. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor suppressor protein. *Proc Natl Acad Sci* USA. **92.** 3046-3050.

Munoz N., Bosch F.X., DeSanjose S., Tafur L., Izarzugaza I., Gili M., Viladiu P., Navarro C., Martos C., Ascunce N., Gonzalez L.C., Kaldor J.M., Guerrero E., Lorincz A., Santamaria M., deRuiz P.A., Aristizabal N., Shah K. 1992. The causal link between human papillomavirus and invasive cervical cancer: a population based study in Columbia and Spain. *Int. J. Cancer.* **52.** 743-749.

Murata M., Iwao K, Miyoshi Y et al. 2001. Activation of the beta catenin gene by interstitial deletions involving exon 3 as an early event in colorectal tumorigenesis. *Cancer lett..***159.** 73-78.

Murthy N.S., Sehgal A, Satyanarayan I, Das DK, Singh V, Das BC, Gupta MM, Mitra AB, Luthra UK. 1990. Risk factors related to biological behavior of precancerous lesions of the uterine cervix. *British J Cancer.* **61.** 732-736.

Muscat J.E., Richie J.P. Jr., Thomson S., Wynder E.L., 1996. Gender differences in smoking and risk for oral cancer. *Cancer Res.* **56.** 5192-5197.

Nagae Y, Kameyama K, Yokoyama M, Naito Z, Yamada N, Maeda S, Asano G, Sugisaki Y, Tanaka S. 2002. Expression of E-cadherin catenin and C-erbB-2 gene products in invasive ductal-type breast carcinomas. *J Nippon Med Sch.* **69 (2).** 165-171.

Nakachi K., Imai K., Hayashi S. Kawajiri K. 1993. Polymorphisms of the CYP1A1 and glutathione-S-Transferase genes associate with susceptibility to lung cancer in relation to cigarette dose in a Japanese population. *Cancer Res.* **53.** 2994-2999.

Naqvi S. H., Wajid S., Mitra A.B. 2004. Restriction fragment length polymorphisms of L1 amplicon using *Rsa* 1 detects five different human papillomavirus types and their co-infections among women attending a gynecological out patient department. *J. Virol. Met.* **117 (1).** 91-95.

Nebert D. W. and Weber W.W. 1990. Pharmacogenetics: In Principles of drug action. *The basis of pharmacology,* 3rd ed.pp. 469-531. Churchil Livingstone Inc. New York.

Negrini M., Monaco C., Vorechovsky I., Ohta M., Druck T., Baffa R. 1996. The *FHIT* gene at 3p14.2 is abnormal in breast carcinomas. *Cancer Res.* **56.** 3173-3179.

Nelson D.R. 1999. Cytochrome P450 and the individuality of species. *Arch Biochem Biophys.* **369**: 1-10.

Nelson D.R. Koymans L., Kamataki T., Stegeman J.J., Feyereisen R., Waxman D.J., Waterman M.R., Gotoh O., Coon M.J., Estabrook R.W., Gunsalus I.C., Nebert D.W. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics.* **6**: 1-42

Nhieu J. T., Renard C. A., Wei Czafrani E. S., Buendia M. A. 1999. Nuclear accumulation of of mutated beta-catenin ion hepatic carcinoma is associated with increased cell proliferation. *Am J Pathology.* **155.** 703-708.

Nieminen P., Aho M., Vesterinen E., Stellato G., Vahen A., Scares V.R.X., Paovonen J. 1991. Natural history of HPV infection preliminary results of a cohort study [abstract] in 1991 papillomavirus workshop, Seattle, W.A. 77.

Nishimura M, Foromoto H., Takeshi K., Kamada M, Toshihiro A. 2000. Microsatellite instability is a late event in the carcinogenesis of uterine cervical cancer. *Gynecologic Oncology.* **79**: 201-206.

Nubia M. F., Xavier Bosch et al. Epidemiologic classification of human papillomavirus type associated with cervical cancer. *New England Journal of Medicine.* 2003; **348**: 518-527.

Nuovo G.J., Crum CP, deVilliers E-M et al. Isolation of a novel human papillomavirus (type 51) from a cervical condyloma. *J Virol.* 1988. **62.** 1452-1456.

Ochiai A., Akimoto S, Kanai Y, Shibata T, Oyama T Hiroshi S. c-erbB-2 gene product associates with catenins in human cancer cells. *Biochem Biophys. Res. Commun.* 1994. **205.** 73-78.

Ohta M., Inoue H., Cotticelli M.G., Kastury K., Baffa R., Palazzo J. 1996. The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell.* **84.** 587-597.

Ohyashiki J.H., OhyashikiK., Aizawa S. 1996. Replication error in hematological neoplasias: Genomic instability in progression of disease is different among different types of leukemia. *Cin. Cancer Res.* **2.** 1583-1589.

Omholt K., Platz A, Ringborg U, Hansson J. Cytoplasmic and nuclear accumulation of beta-catenin is rarely caused by CTNNB1 exon 3 mutations in cutaneous malignant melanoma. *Int J Cancer.* 2001. **92.** 839-842.

Omura T. 1999. Forty years of Cytochrome P450. *Biochem Biophys Res Commun* **266**: 690-698.

Ougolkov A., Mai M., Takahashi Y., Omote K., Bilim V., Shimizu A., Minamoto T. 2000. Altered expression of beta-catenin and c-*erbB*-2 in early gastric cancer. *J Exp Clin Cancer Res.* **19(3)**:349-55.

Oyama T., Kanai Y., Ochiai A., Kimoto .S, Oda T., Yanagihara K., Nagafuchi A., Tsukita S., Shibamoto S., Ito F., Takeichi M., Matsuda H., Hirohashi S. 1994. A truncated β-catenin disrupts the interaction between E-cadherin and β-catenin: a cause of loss of intercellular adhesiveness in human cancer cell lines. *Cancer Res.* **54.** 6282-6287.

Papadopoulos N., Lindblom A. 1997. Molecuylar basis of HNPCC: mutations of MMR genes. *Hum Mut.* **10.** 89-99.

Park J.Y., Muscat J.E., Ren Q., Schantz S.P, Harwhick R.D., Stern J.C., Pike V., Richie J.P. Jr., Lazarus P. 1997. CYP1A1 and GSTM1 polymorphisms and oral cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **6.**791-797.

Patti Kay, Kathleen Meehan, Anna-Lise Williamson. 2002. The use of nested Polymerase chain reaction and restriction fragment length polymorphism for the detection and typing of mucosal HPVs in samples containing low copy number of viral DNA. *J. Virol. Met.* **105** : 159-170.

Paulson T.G., Wright F.A., Parker B.A., Russack V., Wahl G.M. 1996. Microsatellite instability correlates with reduced survival and poor disease prognosis in breast cancer. *Cancer Res.* **56.** 4021-4026.

Peifer M. 1997. β-catenin às oncogene: the smoking gun [comment]. *Science.* **275.**1752-1753.

Pelkonen O., Breimer D.D. 1994. Role of environmental factors in the pharmacokinetics of drugs: Considerations with respect to animal models, P-450 enzymes, and probe drugs. In (Welling PG & Balant LP eds.) *Handbook of Experimental Pharmacology, Springer-Verlag, Berlin, Germany.* **110,** pp 289-332.

Pelkonen O., Mäenpää J., Taavitsainen P., Rautio A., Raunio H. 1998. Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* **28**: 1203-1253.

Peltomaki P., Lothe L. A., Aaltonen L. A. 1993. Microsatellite instability is associated with tumors that characterize the hereditary nonpolyposis colorectal carcinoma syndrome. *Cancer Res.* **53.** 5833-5855.

Peltomaki P., Vasen HFA, Bisgard ML et al. Mutations predisposing to hereditary nonpolyposis colorectal cancer: Database and results of a collaborative study. *Gastroenterology.* 1997. **113.** 1146-1153.

Perucho M. Cancer of the microsatellite mutator phenotype. *J Biol. Chem.* 1996. **377.**675-684.

Pfister H. 1987. Human papillomaviruses and genital cancer. *Adv. Cancer Res.* **48.** 113-147.

Pfister H., Fuchs P.G. 1994. Aanatomy, taxonomy and evolution of papillomaviruses. *Intervirology.* **37.** 143-149.

Phillips N. J., Zeigler M. R., Radford D. M. 1996. Allelic deletion on chromosome 17p13.3 in early ovarian cancer. *Cancer Res.,* **56.** 606-611.

Pierre J.F., Dominique D. 1998. Loss of heterozygosity on the short arm of chromosome 3 in cervical intraepithelial neoplasia without concomitant cervical carcinoma. *Pathobiology..* **66:** 306-310.

Pinion S.B., Kennedy J.H., Miller R.W., Mac Lean, A.B. 1991. Oncogene expression in cervical intraepithelial neoplasia and invasive cancer of cervix. *Lancet.* **337.** 819-820.

Pizzighella, S., Pisoni, G., Bevilacqua, F., Vaona, A., Palu, G., 1995. Simultaneous polymerase chain reaction detection and restriction typing for the diagnosis of human genital papillomavirus infection. *J. Virol. Methods* **55.** 245–256.

Qu W., Jiang G., Cruz Y, Chang C. J., HO G. Y. F., Klein R., Burk R. D.1997. PCR detection of Human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. *J. Clin. Microbiol.* **35 (6).** 1304-1310.

Reagan J.W., Seidemann I.L, Saracusa Y. 1953. Cellular morphology of carcinoma *in situ* and dysplasia or atypical hyperplasia of uterine cervix. *Cancer.* **6**:224-35.

Rhyu M. Park W., Meltzer S. J. 1994. Microsatellite instability occurs frequently in human gastric carcinoma. *Oncogene.* **9.** 29-32.

Richart R.M. 1973. Cervical intraepithelial neoplasia: a review. In : Sommers, S.C., (ed.),, *PathologyAnnual.* East Norwalk, C.T. Appleton-Century-Crofts. pp:301-328.

Risinger J. I., Berchuck A., Kohler M. F. 1993. Genetic instability of microatellites in endometrial carcinoma. *Cancer Res.* **53.** 5100-5103.

Risinger J.I., Umar A., Boyar J. C. 1995. Microsatellite instability in gynecological sarcomas and in *hMSH2* mutants uterine sarcoma cell lines defective in mismatch repair activity. *Cancer Res.* **55.** 5664-5669.

Rodriguez J.A., Barros F., Carracedo A., Mugica C.M. 1998. Low incidence of microsatellite instability in paients with cervical carcinomas. *Diagn. Mol. Pathol.* **7.** 276-282.

Rubinfeld B., Souza B, Albert I, Munemitsu S, Polakis P. 1995. The APC protein and E-cadherin form similar but independent complexes with alpha-catenin, beta-catenin and plakoglobin. *J. Biol. Chem.* **270.** 5549-5555.

Rubinfeld B., Souza B, Albert I, Muller O, Chamberlain SH, Masiarz FR, Munemitsu S, Polakis P. 1993. Association of the APC gene product with β-catenin. *Science.* **262.** 1731-1734.

Saiki R.K.S., Scharf F, Faloona KB, Mullis GT, Horn HA Erlich, Arnheim N. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science.* 1985. **230.** 1350.

Saiki, R.K., Chang, C.A., Levenson, C.H., Warren, T.C., Boehm, C.D., Kazaian, H.H., 1988. Diagnosis of sickle cell anemia and _-thalassemia with enzymatically amplified DNA and non-radioactive allele-specific oligonucleotide probes. *N. Engl. Med.* **319.** 537–541.

Sambrook J., Fritch E.F., Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd ed, New York: Cold Spring harbor Laboratory.

Schiffman M. Latest HPV findings: some clinical implications. *Cont. OB/GYN.* 1993. **38(10).** 27-40.

Schiffman M.H. 1994. Epidemiology of cervical human papillomavirus infections. *Curr. Top. Microbiol. Immunol.* **196.** 54-81.

Schiffman M.H., Kiviat NB, Burk RD,et al.Accuracy and interlaboratory reliability of human papillomavirus DNA testing by hybrid capture. *J.Clin Microbiol.*1995; **33**: 545-550.

Schwarz E., Dürst M, Demankowski G, Lattermann O, Zech E, Wolfsberger E, Suhai S, H zur Hausen. 1983. The sequence and genome organization of genital human papillomavirus type 6b. *EMBO J.* **2.** 2341-2348.

Schwarz E., Freese U.K., Gissmann L., Meyer W., Roggenbuck B., Stremlau A., zur Hausen H. 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature.* **314.** 111-114.

Seedorf K., Krämmer G, Dürst M, Suhai S, Röwekamp W. Human papillomavirus type 16 DNA sequence. *Virology.* 1985. **145.** 181-185.

Semba S., Yokozaki H., Yamamoto S. 1996. Microsatellite instability in precancerous lesions and adenocarcinomas of the stomach. *Cancer.* **77.** 1620-1627.

Seruca R. Santos N. R., David L.1994. Sporadic gastric cancers with microsatellite instability display a particular clinicopathologic profile. *Int J Cancer.* **64.** 32-36.

Shah K. V., and Howley P.M. 1992. Papillomavirus . In : Lennette. E.H., ed. Laboratory Diagnosis of Viral Infection. 2nd .Marcel Dekker, Inc., pp 591-612.

Shanta V, Krishnamurthi S, Gajalakshmi CK, Swaminathan R, Ravichandran K. 2000. Epidemiology of cancer of the cervix: global and national perspective. *J. Indian Med. Assoc.* **98** (2). 49-52.

Sharma A., Pratap M, Malhotra V, Sawhney, Khan I U, Bhambhani S, Mitra A B.Frequent amplification of C-erbB2 (Her-2/nue) oncogene in cervical carcinomas as detected by non fluorescence in situ hybridization technique on paraffin sections. *Oncology.*1999; **56**: 83-87.

Sillins I., Wang Z et al. 1999. Serological evidence for protection by HPV type 6 infection against HPV type 16 cervical carcinogenesis. *Journal of General Virology.* **80**: 2931-2936.

Simon B., Bartsch D., Barth P. 1998. Frequent abnormalities of the putative tumor suppressor gene *FHIT* at 3p 14.2 in pancreatic carcinoma cell lines. *Cancer Res.* **58.** 1583-1587.

Simon D., Knowles BB, Weith A. 1991. Abnormalities of chromosome 1 and loss of heterozygosity on 1p in primary hepatomas. *Oncogene.* **6.** 65-770.

Simons A.M., Phillips D.H., Coleman D.V. 1993. Damage to DNA in cervical epithelium related to smoking tobacco. *Br Med J.* **306.** 1444-1448.

Skalova A., Vanecek T, Losan F, Papoutsidesova, Finek J. 2003.Feb. Detection of HER-2/neu in breast carcinoma. *Cas Lek Cesk.*; **142(2):** 93-8.

Slamon D.J., Clark GM, Wong SG, Levin WJ, Ullrich A, Mc Guire WL. 1987. Human breast cancer: correlation of relapse and survival with amplification of the Her-2/*neu* oncogene. *Science* (Washington DC). **235.** 17-182.

Slamon D.J., Godolphin W, Jones I A, Holt J A, Wong S G, Keith D E, Lewin W J, Stuart S G, Udov J, Ullrich A, Pres M F. 1989. Studies of the Her-2/nue proto-oncogene in human breast and ovarian cancer. *Science.* **244**: 702-712.

Snijders P.J.F., van der Brule AJC et al. 1990.The use of general primers in the polmerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. *J. Gen. Virol.* **71.** 173-181.

Soh L.T., Heng D, Lee IW, Hui KM. 2002 (Sep-Oct). The relevance of oncogenes as prognostic markers for cervical cancer. *Int J Gynaecol Cancer.* **12 (5).** 465-474.

Sood A. K., Skilling J. S., Buller R. E. 1997. Ovarian cancer genomic instability correlates with p53 frameshift mutations. *Cancer Res.* **57.** 1047-1049.

Sozzi G., Veronese ML, Negrini M, Baffa R, Cotticelli MG, Inoue H, Tornielli S, Pilotti S, De Gregorio L, Pastorino U, Pierotti MA, Ohta M, Heubner K, Croce CM. The *FHIT* gene at 3p14.2 is abnormal in lung cancer. *Cell.* 1996. **85.** 17-26.

Strickler J. G., Zheng J., Shu Q. 1994. p53 mutations and microsatellite instability in sporadic gastric cancer: when guardians fail. *Cancer Res.* **54.** 4750-4755.

Takata M, Fujimoto A, Aoki H, Hatta N, Ooi A, Takehara K.1999. *erbB*-2 overexpression but no activation of beta-Catenin gene in extramammary Paget's disease. *J Invest Dermatol.* **113(2):**258-62

Takayama T., Shiozaki H, Shibamoto S, Oka H, Kimura Y, Tamura S, Inoue M, Monden T, Ito F, Monden M. Jan.,1996. Beta catenin expression in human cancers. *American J Pathology.* **148(1).** 39-46.

Takayama T., Shiozaki H., Doki Y., Oka H., Inoue M., Yamamoto M., Tamura S., Shibamoto S., Ito., F., Monden M. 1998. Abberant exprssion and phosphorylation of beta-catenin in human colorectal cancer. *Br J Cancer.* **77.** 605-613.

Takehiro U., Yuchiro D, Hiroyashi T, Hitoshi S, Masahiko Y, Mitsugu S, Shigeyuki T, Takushi Y, Yoshiyuki F, Morito M. 2001 Correlation of beta-catenin and cyclin D1 expression in colon cancers. *Oncology.* **61.** 226-233.

Tetsu O., Mc Cormick F. 1999. Beta catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature.* **398.** 422-426.

Thibodeau S.N., Bren G, Schaid D. 1993. Microsatellite instability in cancer of the proximal colon. *Science.* **260.** 816-819.

Trent J. M., Thompson F. H., Buick R. N. 1985. Generations of clonal variants in a human ovarian carcinoma studied by chromosome banding analysis. *Cancer Genet Cytogenet.* **14.** 153-161.

Trinza Z., Clayman G.L., Spitz M.R., Briggs K.L., Goepfert H. 995. Glutathione-S-Transferase genotypesas risk factors for head and neck cancer. *Amer. J Surg.***170.** 499-501.

Tsuda H., Hirohashi S., Shimosato Y., Hirota T., Tsugane S., Yamamoto H. 1989. Correlation between long term survival in breast

cancer patients and amplification of two putative oncogene amplification units: *list-1/int-2* and *c-erbB-2/ear-1. Cancer Res.* **49.** 3104-3108.

Tsuda H., Hirohashi S., Shimosato Y., Tanaka Y., Hirota T., Tsugane S., 1990. Immunohistochemical study on overexpression of *c-erb-B2* protein in human beast cancer: its correlation with gene amplification and long term survival of patients. *Jpn J Cancer Res.* **81.** 237-32.

Tyson F.L., Boyer CM, Kaufman R, O'Briant K, Cram G, Crews JR, Soper JT, Daly L, Fowler WC, Haskill JS, Bast RC. 1991. Expression and amplification of Her-2/nue (C-erbB2) protooncogene in epithelial ovarian tumours and cell lines. *Am J Obstet Gynecol.* **165.** 640-646.

Uchida T., Wada C., Wang C., Egawa S., Ohtani H., Koshiba K. 1994. Genomic instability of microsatellite repeats and mutations of H-, K-, and N- *ras* and p53 genes in renal cell carcinoma. *Cancer Res.* **54.** 3682-3685.

Ueda M., Gemmil RM, West J. et al. 2001. Mutations of the beta-catenin genes are uncommon in human lung, breast, kidney, cervical and ovarian carcinomas. *Br J Cancer.* **85:** 64-68.

Utsunomiya T., Doki Y., Takemoto H., Shiozaki H., Yano M., SekimotoM., Tamura S., Yasuda T., Fujiwara Y., Monden M. 2001. Correlation of Beta-Catenin and Cyclin D1 Expression in Colon Cancers. *Oncology.* **61**.226-233.

Van den Brule A.J.C., Claas HCJ, du Maine M, Melchers WJG, Helmerhorst T, Quint WGV, Lindeman J, Meijer JLM, Walboomers JMM. 1989.Use of anti-contamination primers in the polymerase chain

reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. *J. Med. Virol.* **29.** 20-27.

Van Ranst M., Kaplan J.B. Burke R.D. 1992. Phylogenetic Classification of Human Pappilomaviruses: Correlation with manifestations. *J. Gen. Virol.* **73.** 2653-2660.

Virgilio L., Shuster M., Gollin S.M., Veronese M.L., Ohta M., Hubner K. 196 *FHIT* gene alterations in head and neck squamous cell carcinomas. *Proc Natl. Acad. Sci. USA.***93.** 9770-9775.

Voeller H. J., Truica C.I., Gelmann E.P. 1998. Beta-catenin mutations in human prostrate cancer. *Cancer Res.* **58:** 2520-2523.

Vogelstein B., Fearon ER, Hamilton SR, Kern Se, Preisinger AC, Leppert M, Nakamura Y., White R, Smits AMM, Bos J.L. 1988. Genetic alterations during colorectal-tumor development. *N Eng J Med.* **319.** 3525-532.

Vogelstein B., Fearon ER, Kern SE, Hamilton SR, , Preisinger AC, Nakamura Y, White R. 1989. Allelotype of colorectal carcinoma. *Science.* **244.** 207-211.

Vousden K. 1993. Interactions of human papillomavirus transforming proteins with products of tumor suppressor genes. *FASEB.***7.**872-879.

Wagata T., Ishizaki K, Imamura M, Shimada Y, Ikenaga M, Tobe T. 1991. Detection of 17p and amplification of the *int-2* gene in esophageal carcinomas. *Cancer Res.* **51.** 2113-2117.

Wahi P. N., Luthra U. K., Mali S., Shimkin M. B. 1972. Prevalence and distribution of cancer of the uterine cervix in Agra district, India. *Cancer.* **30.** 710.

Walboomer J.M.M., Jacobs MV, van Oostreen JW, van der Brule AJC, Snijders PJF, Meijer CJLM: 1997. Detection of genital HPV infections and possible clinical implications. HPV infection in dermatovenerology. 341-364. New York.CRC Press. Boca Raton.

Walboomers J. M. M., Jacobs M. V., Manos M. M., Bosch F. X., Kummer J. A., Shah K. V., Snijders P. J., Peto J., Meijer C.J., Munoz N. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worlwide. *J. Pathol.* **189.** 12-19.

Warwick A. P., Redman C. W. E., Jones P. W. Fryer A. A., Gilford J., Alldersea J., Strange . C. 1994. Progression of cervical intra epithelial neoplasia to cervical cancer: interactions of cytochrome p450 CYP2D6 EM and glutathione-S- Transferase GSTM1 null genotypes and cigarette smoking. *Br J Cancer.* **70.** 704-708.

Watanabe H., Jass J. R., Sobin L. H. 1990. Histological typing of esophageal and gastric tumors. *Ed 2. Berlin, Springer.*

Watanabe M., Imai H., Kato H., Shiraishi T., Ushijima T., Nagao M., Yatani R. 1996. Microsatellite instability in latent prostrate cancers. *Int. J. Cancer.* **69.** 394-397.

Weiss L.K., Kau T.Y. Sparks B.T., Swanson G.M. 1994. Trends in cervical cancer incidence among young black and white women in metropolitan Detroit. *Cancer.***73.** 1849-1854.

Wijnen J., Khan P.M., Vasea H. 1996. majority of *hMLH1* mutations responsible for hereditary nonpolyposis colorectal cancer cluster at the exonic region 15-16. *Am. J. Hum. Genet.***58.** 300-307.

Winkelstein W., 1990. Smoking and cervical cancer.-current status: a review. *Am J Epidemiol.* **131.** 945-9690.

Winkelstein W. 1977. Smoking and cancer of the uterine cervix: hypothesis. *Am J Epidemiol.* **106.** 257-259.

Wistuba I.I., Montellano FD, Milchgrub S, Virmani AK, Behrens C, Chen H, Ahmadian M, Nowak JA,Muller C, Minna JD, Gazdar AF. 1997. Deletions of chromosome 3p are frequent and early events in the pathogenesis of uterine cervical carcinoma. *Cancer Research.* **57.**3154-3158.

Wolf C. R., Smith C.A.D., Gough A.C., Moss J. E., Vallis K.A., Howard G., Carey F. J., Mills K., McNee W., Carmichael J., Spurr N. K. 1992. Relationship between the debrisoquine polymorphism and ancer susceptibility. *Carcinogenesis.* **13.** 1035-1038.

Wong A.J., Ruppert JM, Eggleston J, Hamilton SR, Baylin SB, Vogelstein B. 1986. Gene amplificaion of *c-myc* and *N-myc* in small cell carcinoma of the lung. *Science* (Washington DC). **233.** 461-464.

Wong Y. F., Chung T.K., Cheung T.H., Tam P.O., Chang A.M. 1997. requent loss of heterozygosity of chromosome 3 short arm detected by PCR-based microsatellite polymorphisms in cervical squamous cell carcinoma. *Cancer Lett.* **115.** 161-164.

Wooster R., Cleton Jenson A. M., Collins N. 1994. Instability of short tandem repeats (microsatellites) in human cancers.*Nature Genet.* **6.**152-156.

Wu M., Shew J., Shun C. 1997. Infrequent *hMSH2* mutations in sporadic gastric adenocarcinoma with microsatellite instability. *Cancer Lett.* **112.** 161-166.

Yee C.J., Roodi N., Verrier C.S. 1994. Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Res.* **54.** 1641-1644.

Yokota J., Tsukada Y, Nakajima T,Gotoh M, Shimosato Y, Mori N, Tsunokawa Y, Sugimura T, Terada M. 1989. Loss of heterozygosity on the short arm of chromosome 3 in carcinoma of the uterine cervix. *Cancer Research.* **49.** 3598-3601.

Yokota J., Yamamoto T, Miyajima N, Tiyoshima K, Nomura N, Sakamoto H, Yoshida T, Tereda M, Sugimora T. 1988. Genetic alterations of the C-*erbB*-2 oncogene occur frequently in tubular carcinoma of the stomach and are often accompanied by amplification of the V erb A homologue. *Oncogene.* **2**: 283-287.

Yutaka Kimura, Shizoki H, Doki Y, Yamamoto M, Utsunomiya T, Kawanishi K, Fukuchi N, Inoue M, Tsujinaka T, Monden M. 1999. Cytoplasmic beta-catenin in esophageal cancers .*Int. J Cancer.* **84.** 174-178.

Zhang X., Silva E, Gerslenson D, Hung MC. 1989. Amplification and rearrangement of *cerbB2* protooncogene in cancer of human genital tract. *Oncogene.* **4;** 985-989.

Zhou X.P., Hoang JM, Cottu PH et al. 1997. Allelic profiles of mononucleotide repeat microsatellites in control individuals and in colorectal tumours with an without replication errors. *Oncogene.* **15;** 1713-1718.

Zou T.T., Lei J., Shi Y.Q., Wang S., Souza R.F., Kong D., Shimada Y., Smolinski K.N., Greenwald BD, Abraham J.M., Harpaz N., Meltzer S.J. 1997. *FHIT* alterations in esophageal cancer and ulcerative colitis (UC). *Oncogene.* **15.** 101-105.

zur Hausen H. 1991.Viruses in human cancers. *Science.* **254.**1167-1163.

zur Hausen H. 1994. Molecular pathogenesis of cancer of the uterine cervix and its causation by specific human papillomavirus type. *Curr Top. Microbiol. Immunol.* **186.** 131-156.

zur Hausen H. 1996. Papillomavirus infections: a major cause of human cancers. *Biochem. Biophys. Acta.* 1288: 55-78.

Master Charts

Chart 1 Showing microsatellite instability with different primers

SN	Case No	Patient	Age	HPV		6	11	16	18	BAT25	BAT26	D2S123	D5S346	D17S250	STATUS
1	100492.1	Rehmati	50	P	I	P	P	N	N	●	○	○	●	●	IMSS
2	270392.5	Pyari Devi	58	P	I	P	P	P	N	○	○	●	○	○	IMSS
3	190791.1	Phoola Devi	56	P	I	N	N	N	N	○	○	○	●	●	IMSS
4	280292.4	Vishambri	50	P	I	P	N	N	N	○	○	○	●	○	IMSS
5	100492.6	Farida	40	P	I	N	N	P	N	●	●	●	●	●	IMSI-H
6	100492.3	Jallo Devi	42	P	I	N	N	N	N	○	●	○	●	●	IMSI-L
7	100492.4	Kamla	40	P	I	P	N	P	N	●	○	○	○	○	IMSS
8	60392.2	Ram Devi	50	P	I	P	N	P	N	○	○	○	○	○	IMSS
9	280292.5	Basanti	47	P	I	P	N	N	N	●	○	○	●	●	IMSI-L
10	240492.2	Shanti	45	P	I	P	N	N	N	○	○	●	○	○	IMSS
11	280292.1	Ramwati	40	P	I	N	N	N	N	○	○	●	●	●	IMSS
12	60392.5	Ratna Devi	40	P	I	P	N	N	N	●	●	●	○	●	IMSI-H
13	280292.3	Ram Dali	45	P	I	P	P	P	N	○	○	○	○	○	IMSS
14	270392.1	Santosh	40	N	I	P	P	N	N	○	○	○	○	●	IMSS
15	270392.3	Gurdeep	58	N	I	N	N	N	N	●	○	○	○	●	IMSS
16	270392.4	Poonam evi	55	N	I	N	N	N	N	●	○	○	○	○	IMSI-L
17	280892.4	Ramka	55	P	I	P	N	N	N	○	○	●	○	○	IMSI-L
18	250992.5	Chand Kaur	45	N	I	N	N	N	N	●	○	●	○	●	IMSS
19	250992.2	Shakuntala	30	P	I	P	N	N	N	○	○	●	●	○	IMSI-L
20	280292.1	Veervati	40	N	I	N	N	N	N	●	○	●	○	●	IMSS
21	80193.6	Joginder Kaur	60	P	I	P	N	N	N	○	○	○	●	●	IMSS
22	10193.3	Har Kaur	55	P	I	P	N	P	N	○	○	●	●	●	IMSS
23	180992.2	Munni	45	P	I	N	N	P	N	○	○	○	○	●	IMSS
24	240792.2	Janaki Devi	54	P	I	P	N	P	N	●	○	○	●	○	IMSI-L
25	120791.7		45	N	I	P	N	N	N	○	○	●	○	●	IMSI-L
26	250992.1	Ramkali	80	P	I	P	N	N	N	○	○	○	○	●	IMSS
27	80193.2	Sulochana	36	P	I	P	N	N	N	●	○	○	○	●	IMSI-L
28	80193.1	Urmila	50	P	I	N	N	P	N	○	○	●	●	●	IMSS
29	20802.2	Nuran	48	P	I	P	P	P	N	●	●	●	○	●	IMSI-L
30	50802.2	Laxmi	52	P	I	P	P	P	N	○	●	●	●	●	IMSS
31	90802.2	Jagmati	44	P	I	N	P	N	N	●	●	●	○	●	IMSI-H
32	60902.1	Akhtari	56	P	I	P	N	P	N	○	●	●	●	●	IMSI-L

Continued

SN	Case No	Patient	Age	HPV	6	11	16	18	BAT25	BAT26	D2S123	D5S346	D17S250	STATUS
33	60902.2	Murty	62	P	N	P	N	N	○	●	●	●	●	MSS
34	60902.3	Shahida	45	P	N	P	P	N	○	●	○	○	●	MSS
35	200902.1	Jagwati	51	P	P	N	P	N	●	●	○	○	●	MSI-L
36	200902.5	Brahmo Devi	58	P	N	N	P	N	○	●	○	●	●	MSS
37	270902.2	Chaman Kali	49	P	P	P	P	N	○	●	●	●	●	MSS
38	11102.2	Shanti	57	P	P	P	P	N	●	●	●	●	●	MSI-H
39	151102.2	Jaideva	45	P	N	P	P	N	○	●	●	●	●	MSI-H
40	151102.3	Omwati	54	P	P	N	N	N	○	●	○	○	●	MSI-L
41	151102.4	Krishna	55	P	P	N	P	N	○	○	●	○	○	MSS
42	151102.5	Sumitra	63	P	P	N	P	N	●	○	○	○	●	MSI-L
43	221102.1	Bimlesh	80	P	P	N	N	N	●	○	●	○	●	MSI-L
44	221102.2	Sakimo	36	P	P	N	P	N	○	○	○	○	○	MSS
45	221102.3	Chandri	50	P	N	N	P	N	●	●	●	○	●	MSI-L
46	291102.1	Aquila	50	P	N	N	P	N	○	○	●	○	●	MSS
47	291102.2	SitaDevi	60	P	P	N	N	N	○	○	○	○	●	MSI-L
48	291102.3	Shiv Dulari	36	P	P	P	P	N	●	○	●	○	●	MSI-L
49	291102.4	Kamla Devi	50	P	P	N	P	N	○	○	●	○	●	MSS
50	91202.1	Maya	50	N	N	N	N	N	○	○	●	○	○	MSI-L
51	131202.1	Bholi Devi	63	P	P	N	N	N	●	○	○	○	○	MSI-L
52	131202.2	Parwati	40	P	P	N	P	N	○	○	●	●	●	MSI-L
53	11102.1	Savitri	42	P	N	N	P	N	●	○	●	○	○	MSI-L
54	181002.1	Shiva Devi	57	P	N	N	N	N	○	○	●	○	●	MSI-L
55	270902.4	Rama Devi	40	P	N	N	P	N	○	●	●	○	○	MSI-H
56	270902.3	Kaniz Khatoon	42	P	P	N	N	N	●	○	○	○	●	MSI-H
57	200902.4	Salma Khatoo	40	P	P	N	N	N	○	○	●	○	○	MSS
58	200902.2	Chameli	50	N	N	N	P	N	●	○	●	○	●	MSI-H
59	130902.3	Kishni	50	P	N	N	P	N	○	○	●	○	●	MSS
60	90802.1	Kasturi	65	P	N	N	P	N	○	●	○	●	●	MSS
61	20802.1	Ketuka	70	P	N	P	P	N	●	○	○	○	●	MSS
62	220702.1	Chandrakali	57	N	N	N	N	N	○	○	●	○	○	MSI-L
63	60502.1	Nahno Devi	70	P	P	N	P	N	●	○	●	○	●	MSI-L
64	80402.3	Jhalo Devi	55	P	N	N	P	N	○	●	○	○	●	MSS
65	80402.1	Rasheedan	55	P	P	N	N	N	●	○	○	○	●	MSI-L
66	10402.1	Balmati	70	P	N	N	P	N	●	○	●	○	●	MSI-H

Continued

SN	Case No	Patient	Age	HPV	6	11	16	18	BAT25	BAT26	D2S123	D5S346	D17S250	STATUS
67	180302.1	Phoolwati	65	P	P	N	N	N	○	●	●	○	○	MSI-L
68	110302.2	Sushila	43	P	P	P	P	N	○	○	●	○	●	MSI-H
69	110202.1	Shakuntala	65	P	N	N	P	N	●	○	○	○	●	MSS
70	40302.3	Savitri	50	P	N	N	P	N	○	○	○	●	●	MSS
71	40302.2	Bano	38	P	N	N	P	N	●	○	●	○	●	MSI-L
72	40302.1	Sanjha	55	P	N	P	P	N	○	●	○	○	○	MSS
73	250202.2	Tari Devi	55	P	P	N	P	N	○	○	●	○	●	MSI-L
74	180202.2	Shingari Devi	65	P	N	N	P	N	●	○	●	○	●	MSI-L
75	180202.1	Meera	32	P	P	N	P	N	●	●	●	○	●	MSI-H
76	110202.1	Urmila	50	P	P	N	N	N	○	○	○	○	○	MSS
77	181201.1	Sheela	55	P	N	N	P	N	●	○	●	○	●	MSI-L
78	41201.1	Satya Bhama	65	N	N	N	N	N	○	○	●	○	●	MSI-L
79	201101.1	Vimla Sharma	50	P	N	P	P	N	○	○	○	○	●	MSS
80	231001.1	Bimla	44	P	N	N	P	N	○	○	○	●	○	MSS
81	161001.2	Leelawati	48	P	P	N	P	N	○	○	●	○	●	MSS
82	161001.1	Amri Devi	60	P	N	N	P	N	○	○	●	●	●	MSI-H
83	110901.1	Ramnandi	55	P	P	N	N	N	○	●	●	○	○	MSI-L

○ Homozygous, MSI -ve and non-informative for LOH study
● Heterozygous MSI -ve and Informative for LOH study
● Homozygous and MSI +ve
● Heterozygous and MSI +ve not showing LOH
● Showing LOH
P = Positive
N = Negative

Chart 2 Showing loss of heterozygosity with different primers

SN	Case No.	Patient	Age	HPV	6	11	16	18	D3S1234	D3S1300	D3S1313	D5S208	D5S406
1	100492.1	Rehmati	50	P	P	P	N	N	●	O	●	●	●
2	270392.5	Pyari Devi	58	P	P	P	P	N	●	O	●	nd	nd
3	190791.1	Phoola Devi	56	P	N	N	N	N	●	●	●	●	O
4	280292.4	Vishambri	50	P	P	N	N	N	●	O	●	O	●
5	100492.6	Farida	40	P	N	N	P	N	O	O	●	O	●
6	100492.3	Jallo Devi	42	P	N	N	N	N	●	●	●	●	●
7	100492.4	Kamla	40	P	P	N	P	N	●	O	●	●	●
8	60392.2	Ram Devi	50	P	P	N	P	N	●	O	●	nd	nd
9	280292.5	Basanti	47	P	P	N	N	N	●	●	●	●	●
10	240492.2	Shanti	45	P	P	N	N	N	●	O	●	O	●
11	280292.1	Ramwati	40	P	N	N	N	N	O	O	●	O	O
12	60392.5	Ratna Devi	40	P	P	N	N	N	O	●	●	O	●
13	280292.3	Ram Dali	45	P	P	P	P	N	O	●	●	nd	nd
14	270392.1	Santosh	40	N	P	P	N	N	●	●	●	●	O
15	270392.3	Gurdeep	58	N	N	N	N	N	●	●	●	O	●
16	270392.4	Poonam evi	55	N	N	N	N	N	●	●	●	●	●
17	280892.4	Ramka	55	P	P	N	N	N	●	●	●	O	●
18	250992.5	Chand Kaur	45	N	N	N	N	N	●	●	●	●	●
19	250992.2	Shakuntala	30	P	P	N	N	N	●	●	●	●	●
20	280292.1	Veervati	40	N	N	N	N	N	●	●	●	O	●
21	80193.6	Joginder Kaur	60	P	P	N	N	N	●	O	●	●	O
22	10193.3	Har Kaur	55	P	P	N	P	N	●	●	●	O	●
23	180992.2	Munni	45	P	N	N	P	N	●	●	●	●	●
24	240792.2	Janaki Devi	54	P	P	N	P	N	●	●	●	O	●
25	120791.7		45	N	P	N	N	N	●	O	●	nd	nd
26	250992.1	Ramkali	80	P	P	N	N	N	●	O	●	O	●
27	80193.2	Sulochana	36	P	P	N	N	N	●	●	●	O	O
28	80193.1	Urmila	50	P	N	N	P	N	●	●	●	●	O
29	20802.2	Nuran	48	P	P	P	P	N	O	●	●	●	●
30	50802.2	Laxmi	52	P	P	P	P	N	●	O	●	●	O
31	90802.1	Jagmati	44	P	N	P	N	N	●	O	●	●	O
32	60902.1	Akhtari	56	P	P	N	P	N	O	●	●	●	●

Continued

SN	Case No.	Patient	Age	HPV	6	11	16	18	D3S1234	D3S1300	D3S1313	D5S208	D5S406
33	60902.2	Murty	62	P	N	P	N	N	○	○	●	●	○
34	60902.3	Shahida	45	P	N	P	P	N	●	○	●	●	●
35	200902.1	Jagwati	51	P	P	N	P	N	○	○	●	●	○
36	200902.5	Brahmo Devi	58	P	N	N	P	N	●	●	●	●	●
37	270902.2	Chaman Kali	49	P	P	P	P	N	○	○	●	●	○
38	11102.2	Shanti	57	P	P	P	P	N	●	●	○	●	●
39	151102.2	Jaideva	45	P	N	P	P	N	○	●	●	●	●
40	151102.3	Omwati	54	P	P	N	N	N	○	○	●	●	○
41	151102.4	Krishna	55	P	P	N	P	N	●	○	○	●	●
42	151102.5	Sumitra	63	P	P	N	P	N	●	●	○	●	○
43	221102.1	Bimlesh	80	P	P	N	N	N	○	○	●	●	●
44	221102.2	Sakimo	36	P	P	N	P	N	●	●	●	●	○
45	221102.3	Chandri	50	P	N	N	P	N	○	●	●	●	●
46	291102.1	Aquila	50	P	N	N	P	N	●	●	●	●	●
47	291102.2	SitaDevi	60	P	P	N	N	N	●	○	●	●	●
48	291102.3	Shiv Dulari	36	P	P	P	P	N	○	●	○	●	●
49	291102.4	Kamla Devi	50	P	P	N	P	N	○	○	●	○	○
50	91202.1	Maya	50	N	N	N	N	N	●	●	●	●	●
51	131202.1	Bholi Devi	63	P	P	N	N	N	●	●	●	●	●
52	131202.2	Parwati	40	P	P	N	P	N	●	○	●	●	●
53	11102.1	Savitri	42	P	N	N	P	N	○	●	●	○	○
54	181002.1	Shiva Devi	57	P	N	N	N	N	○	●	○	●	○
55	270902.4	Rama Devi	40	P	N	N	P	N	●	○	●	○	●
56	270902.3	Kaniz Khatoon	42	P	P	N	N	N	●	●	●	●	●
57	200902.4	Salma Khatoon	40	P	P	N	N	N	○	●	●	●	●
58	200902.2	Chameli	50	N	N	N	P	N	○	●	●	○	●
59	130902.3	Kishni	50	P	N	N	P	N	●	○	●	●	●
60	90802.1	Kasturi	65	P	N	N	P	N	●	○	●	●	●
61	20802.1	Ketuka	70	P	N	P	P	N	●	●	●	○	○
62	220702.1	Chandrakali	57	N	N	N	N	N	○	○	○	●	●
63	60502.1	Nahno Devi	70	P	P	N	P	N	○	●	●	●	●
64	80402.3	Jhalo Devi	55	P	N	N	P	N	●	●	●	●	●
65	80402.1	Rasheedan	55	P	P	N	N	N	○	○	●	○	●
66	10402.1	Balmati	70	P	N	N	P	N	●	○	●	●	○

Continued

SN	Case No.	Patient	Age	HPV	6	11	16	18	D3S1234	D3S1300	D3S1313	D5S208	D5S406
67	180302.1	Phoolwati	65	P	P	N	N	N	●	●	●	●	○
68	110302.2	Sushila	43	P	P	P	P	N	●	●	○	○	●
69	110202.1	Shakuntala	65	P	N	N	P	N	○	○	●	●	●
70	40302.3	Savitri	50	P	N	N	P	N	●	●	●	●	○
71	40302.2	Bano	38	P	N	N	P	N	●	○	●	●	●
72	40302.1	Sanjha	55	P	N	P	P	N	○	●	●	●	●
73	250202.2	Tari Devi	55	P	P	N	P	N	●	●	○	○	●
74	180202.2	Shingari Devi	65	P	N	N	P	N	●	●	●	○	●
75	180202.1	Meera	32	P	P	N	P	N	○	○	●	●	○
76	110202.1	Urmila	50	P	P	N	N	N	●	○	●	●	●
77	181201.1	Sheela	55	P	N	N	P	N	●	●	○	○	●
78	41201.1	Satya Bhama	65	N	N	N	N	N	○	○	●	○	●
79	201101.1	Vimla Sharma	50	P	N	P	P	N	○	●	●	○	○
80	231001.1	Bimla	44	P	N	N	P	N	●	●	○	●	○
81	161001.2	Leelawati	48	P	P	N	P	N	●	○	●	●	●
82	161001.1	Amri Devi	60	P	N	N	P	N	○	●	●	●	○
83	110901.1	Ramnandi	55	P	P	N	N	N	○	○	●	●	●

○ Homozygous, MSI -ve and non-informative for LOH study
● Heterozygous MSI -ve and Informative for LOH study
● Homozygous and MSI +ve
● Heterozygous and MSI +ve not showing LOH
● Showing LOH
P = Positive
N = Negative

HPV 16 = 48/83 = 58%
HPV 6/16 = 23/83 = 28%
MEDIAN AGE 50 Yrs

Chart 3 showing expression of *erbB-2* and *beta catenin* in squamous cell carcinoma of uterine cervix

S.No.	Slide No.	Histology	HPV	*erbB-2*	*β-catenin*
1	H/2002-145	MD	16+	(-)	+
2	H/2001-41	MD	+	(-)	±
3	H/2001-52	MD	+	(+)	±
4	H/2001-149	PD	+	(-)	+
5	H/2001-322	MD	16+	(++)	±
6	H/2001-428	WD	+	(-)	±
7	H/2001-434	MD	+	(-)	+
8	H/2001-436	MD	+	(-)	±
9	H/2001-437	WD	16+	(++)	±
10	H/2001-324	WD	16+	(++)	+
11	H/2001-375	WD	-	(-)	±
12	H/2001-389	WD	+	(-)	+
13	H/2001-398	WD	16+	(-)	±
14	H/2001-411	WD	16+	(-)	±
15	H/2001-426	WD	-	(-)	±
16	H/2001-424	WD	16+	(++)	±
17	H/2001-415	PD	+	(-)	+
18	H/2001-414	MD	16+	(+)	±
19	H/2001-413	PD	+	(-)	+
20	H/2002-285	MD	16+	(++)	+
21	H/2002-270	MD	+	(-)	+
22	H/2002-238	WD	-	(-)	+
23	H/2002-256	MD	+	(-)	+
24	H/2002-246	WD	16+	(+)	±
25	H/2002-220	MD	+	(-)	+
26	H/2002-219	MD	16+	(-)	+
27	H/2002-203	WD	-	(-)	+
28	H/2002-204	MD	16+	(++)	±

(+) = Weakly Positive
(++) = Strongly Positive
(-) = Negative
+ = HPV Positive by MY09/11 Primers
16+ = HPV Type 16 Positive
PD = Poorly differentiated squamous cell carcinoma
MD = Moderately differentiated squamous cell carcinnoma
WD = Well differentiated squamous cell carcinoma
+ = Preserved Expression
± = Reduced Expression

Chart 4 showing expression of *erbB-2* and *beta catenin* in adenocarcinoma of uterine cervix

S.No.	Slide No.	*erbB-2*	*β-catenin*
1	H/97-18	(-)	±
2	H/97-67	(-)	+
3	H/97-81	(+)	+
4	H/97-83	(++)	±
5	H/97-106	(-)	+
6	H/97-115	(-)	+
7	H/97-128	(-)	+
8	H/97-177	(-)	±
9	H/97-199	(-)	+
10	H/97-201	(-)	±
11	H/96-222	(-)	+
12	H/96-237	(-)	+
13	H/96-241	(+)	+
14	H/96-246	(+)	+
15	H/96-269	(-)	±
16	H/96-270	(-)	+
17	H/96-277	(++)	±
18	H/96-280	(-)	+
19	H/96-324	(-)	+
20	H/96-328	(-)	+
21	H/96-313	(+)	±
22	H/2001-412	(+)	+

(+) = Weakly Positive
(++) = Strongly Positive
(-) = Negative
\+ = Preserved Expression
± = Reduced Expression

Publication

Available online at www sciencedirect com
SCIENCE DIRECT
Journal of Virological Methods 117 (2004) 91–95
Journal of Virological Methods
www elsevier com/locate/jviromet

Short communication

Restriction fragment length polymorphism of L1 amplicon using Rsa 1 detects five different human papillomavirus types and their co-infections among women attending a gynaecological outpatient department

Samar Husain Naqvi[1], Saima Wajid[1], Ardhendu B. Mitra*

Institute of Cytology and Preventive Oncology Indian Council of Medical Research Maulana Azad Medical College Campus Bahadurshah Zafar Marg New Delhi 110002 India

Received 27 August 2003, received in revised form 3 December 2003 accepted 4 December 2003

Abstract

Detection of human papillomavirus (HPV) types 6, 11, 16, 18, and 33 including co-infections among females attending gynaecological outpatient department and cancer clinics, was done by restriction fragment length polymorphism (using Rsa-1), of approximately 450 bp amplicon, obtained by the amplification of the L1 region of HPV genome with consensus primers MY09/11 [Cancer Cells 7 (1989) 209] The results were further tested with HPV type specific primers [J Med Virol 29 (1989) 20] The technique was found to be low-cost and less time consuming The advantage of Rsa 1 over other enzymes was that it detects the five most prevalent HPV types commonly associated with warts, cervical dysplasia, and cancer

Keywords HPV, RFLP, Rsa 1, Cervical dysplasia, Cancer

1. Method

(i) DNA extraction = 3 h
(ii) Polymerase chain reaction for detection of HPV infection by MY09/11 primers = 2 h
(iii) Electrophoresis = 45 min
(iv) Preparation of polyacrylamide gel = 1 h
(v) RE digestion = 1 h
(vi) Electrophoresis on polyacrylamide gel = 2 5 h
(vii) Staining with ethidium bromide = 25 min Total time in detection = 11 h

Human papillomaviruses (HPV) are small non-enveloped tumorigenic double stranded DNA viruses of about 8 kbp More than 100 HPV types have been identified and characterized on the basis of DNA sequences Human papillomaviruses cause warts (Schwarz et al , 1983), papillomas (Gissmann et al , 1982) and certain cancers including cancer of the uterine cervix (Bosch et al , 1995, Beaudenon et al , 1986, Cole and Streeck, 1986) Cervical intraepithelial neoplasia and cervical cancer both are strongly associated with persistence presence of some types of human papillomavirus Over 90% of the cervical carcinoma have been shown to contain some high-risk HPV types (Nieminen et al , 1991) The genital HPVs are classified into two groups high and low-risk based on whether or not the lesions with which they are associated are at significant risk for malignant progression The low-risk HPVs such as HPV6 and 11 are generally associated with genital warts (condyloma accuminata and flat genital warts), lesions that only rarely progress to cervical cancer The high-risk viruses, such as HPV16 (Favre et al , 1989, Seedorf et al , 1985), HPV18 (Cole and Danos, 1987) and HPV33 (Beaudenon et al , 1987, Cole and Streeck, 1986), and few other are associated with carcinoma in situ and cervical cancer HPV16, 18, 33, and 45 account for more than 90% of the high-risk types The MY09/MY11 primer set mediated PCR (MY-PCR) (Hildesheim et al , 1994, Manos et al , 1989) and the GP5 + GP6 + primer set mediated PCR (GP + PCR) (de Roda Husman et al , 1997, Jacobs

* Corresponding author Tel +91-11-23221596/91-9811324553, fax +91-11 2323340C
E-mail address ab_mitra@rediffmail com (A B Mitra)
[1] Senior Research Fellows, Department of Science and Technology, India Grant No SP/SO/B68/97

0166 0934/$ – see front matter © 2003 Elsevier B V All rights reserved
doi 10 1016/j jviromet 2003 12 002

et al., 1999) are the most frequently used amplification systems for the detection of HPV DNA in clinical samples and genotyping by type specific primers (Van den Brule et al., 1989) There was differential amplification sensitivity of HPV types between the two systems MY-primer set was found to be inefficient for HPV type 35 detection but was proved to be more sensitive than GP + PCR primer set in the amplification of multiple HPV DNA types within a given sample (Qu et al., 1997). According to a recent meta-analysis (Clifford et al., 2003), it was found that HPV prevalence vary little between geographical regions. The most common high-risk HPV types in cervical squamous cell carcinoma was HPV16 whereas, HPV18 was predominant in adenocarcinomas and adenosquamous carcinomas followed by HPV16. The MY09/11 primer set has been used predominantly in epidemiologic studies in north and south America and Asia (Bosch et al., 1995; Liaw et al., 1995) whereas the GP5 + GP6 has been used primarily in Europe (de Rod Husman et al., 1995). Furthermore, various studies indicated frequent co-infections of HPV16 and HPV6, suggesting interaction between different HPV types in multiple infections. HPV16 and 6 appeared to have antagonistic interference in cervical carcinogenesis. Serological evidences indicate protection by HPV type 6 infection against HPV type 16 cervical carcinogenesis (Sillins et al., 1999). whereas in co-infections the E6 protein of low-risk HPV type 6 augments the ability of E7 of HPV type 16 or E7 of HPV type 6 coupled with E6 of type 16 increases the potency and ability to immortalize cells (Halbert et al., 1992) These studies suggest the detection of low-risk HPV types and their co-infections along with HR-HPVs to be important for understanding the pathogenesis of the disease and the biological behavior of HPVs during cervical carcinogenesis. Infection of multiple HPV DNA types is a risk factor for cervical intraepithelial neoplasia (Liaw et al., 1995) These observations demand for an early detection of virus types, especially those associated with cervical dysplasia and cancers, with an easy, cost-effective and simple diagnostic procedure. We, developed a new rapid and cost-effective PCR–RFLP technique, using restriction digestion with Rsa 1, of the amplified products of L1 region (by MY09/11 consensus primers). It detects five different HPV types and their co-infections simultaneously (Table 1), commonly associated with warts, precancerous, and cancerous lesions. Cervical scrapes from the women, attending the outpatient department of Gynaecology, were obtained after taking their proper consent. Scrapes were taken by scraping the ectocervix or the surface of the portio with Ayer's spatula and put in 5 ml of 1 × PBS (phosphate buffer saline) The samples were transported to the laboratory at 4 °C and stored at −20 °C till further processing. The DNA isolation from the exfoliated cervical cells involves basically two steps: first washing the pellet twice with tris–triton buffer (TTB) and PK digestion in tris–EDTA buffer (TEB) (Gopalkrishna et al., 1992), total time required in DNA isolation was 3 h. Cervical scrapes were agitated along with the wooden spatula, and poured in 1.5 ml tubes after removing the spatula. Then the tubes were centrifuged at 2000 rpm for 2 min at 4 °C. The pellet was rewashed with chilled 1 × PBS and cold centrifugation was done for 2 min at 2000 rpm The pellet was washed twice in 1 ml chilled TTB (10 mM Tris–HCl pH 8.0), 10 mM $MgCl_2$, 300 mM Sucrose and 0.8% Triton-X 100). The pellet was collected and rewashed in 0.5 ml cold TE buffer (10 MM Tris–HCl (pH 8.0), 10 mM EDTA and 10 mM NaCl). Finally, the pellet was resuspended in 200 μl TE buffer supplimented with Proteinase K (PK = 1.25 mg/ml) and incubated at 65 °C for 2.5 h. The tubes were shaken vigorously every half hour in order to allow uniform analysis of the pellet till the solution became transparent. PK inactivation was done by boiling at 95 °C for 10 min. β-Globin primers PC04 (5′-CAACTTCATCCACGTTCACC-3′) and GH20 (5′-GAAGAGCCAAGGACAGGTAC-3′) (Saiki et al., 1988), which produces a 268 bp PCR product, were used as an internal amplification control. Precautions were taken to avoid cross contamination of samples and the amplified products. The samples were then screened for the presence of HPV using proper controls. The detection of HPV was done by PCR amplification of the L1 region of HPV genome using degenerate primers, MY09/11 (Manos et al., 1989) (Fig. 1). PCR using MY09/11 primers have been proved to be a valid tool for the detection of a wide spectrum of genital HPVs (Gravitt et al., 1998) The amplification was done using Taq DNA polymerase (0.6 U/25 μl, Catalog No. MME 5J), 10 × buffer with 2.5 $MgCl_2$ and dNTPs 200 μM/25 μl (UK Biolab; Catalog No. NO442S), with initial denaturation of 95 °C for 4 min followed by 35 cycles of 94, 56, and 72 °C each for 30 s and a final extension of 72° for 7 min. Eight microliters of the sample was loaded for electrophoresis on 2% agarose (Sigma; Catalog No. A9539), stained with ethidium bromide, for the screening of HPV positive cases (Fig 1) RFLP of the PCR products of all the HPV positive samples was done using 1 U of Rsa 1 (UK Biolab; Catalog No 167 L) with NEB reaction buffer, and incubating at 37 °C for 1 h After digestion the products were resolved on 8% polyacrylamide non-denaturing gel with TBE buffer, after 30 min of staining with ethidium bromide, the products were visualized under UV/gel documentation system (Bio Rad) using the software quantity one. The bands show the types of HPV present including their co-infections (Fig 2)

The degenerate primers MY09/11 used for amplifying L1 region, accepts certain degree of mismatch between primers and target DNA, accomplished by reducing the stringency of primer annealing, there being always a difference of few bases in the amplicon for different HPV types, which are not distinguishable in lower concentration of agarose gels The product (amplicon) sizes amplified by MY09/11 primers for HPV type 6 was 447 bp; HPV11–449 bp, HPV16–452 bp, HPV18–455 bp, and for HPV33 was 449 bp, which after digestion with Rsa 1 (recognition sequence GT′AC) gives the following band sizes (also see Fig 2)

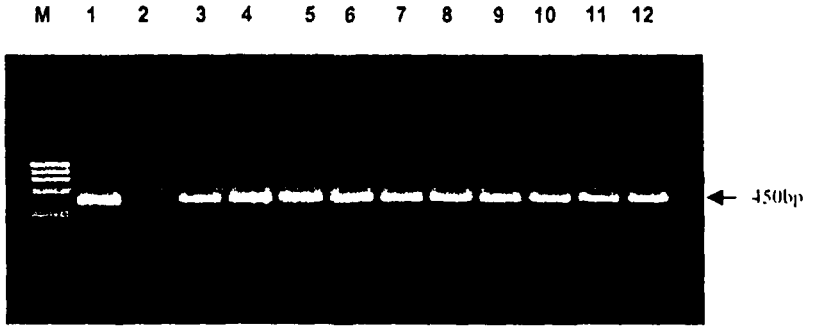


Fig. 1. Representative photograph showing HPV Positive cases amplified using MY09/11 consensus primers (amplicon size ~450 bp) resolved on 2% agarose with ϕX174 (Hae III digested) marker. Lanes 1 and 2 are positive and negative controls, respectively, 3 and 4 mild dysplasia, 5–7 severe dysplasia, 8–11 squamous cell carcinoma and 12 adenocarcinoma of uterine cervix.

Table 1
Shows the number of possible cuts and the size of the fragments resolved after the digestion of L1 amplicon with Rsa I for different HPV types

Type	No. of cuts	Fragment size
HPV 6	3	72, 67, 149, 159
HPV 11	3	72, 216, 26, 135
HPV 16	2	72, 70, 310
HPV 18	4	72, 38, 85, 125, 135
HPV 33	3	72, 39, 236, 102

The bold values indicate the distinguishing bands

To confirm the results obtained by RFLP, all positive cases were amplified using type specific primers (Table 2). 1–2 μl DNA was used for amplification with type specific primers, temperature profile for the amplification was same for each type, initial denaturation 95 °C for 5 min, followed by 35 cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 7 min. The products

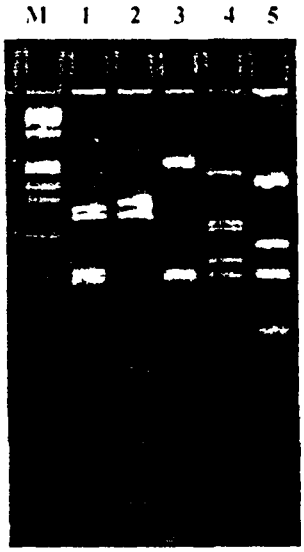


Fig. 2. Rsa I digest of L1 amplicon resolved on 8% PAGE showing HPV types 6, 11, 16, 18, and 33 in lanes 1–5, respectively (M, ϕX174 (Hae III digested) marker).

Table 2
Primers used for PCR

HPV type	Primer sequence
Type 6 (F)	5′-TAGTGGGCCTATGGCTCGTC-3′
(R)	5′-TCCATTAGCCTCCACGGGTG-3′
Type 11 (F)	5′-GGAATACATGCGCCATGTGG-3′
(R)	5′-CGAGCAGACGTCCGTCCTCG-3′
Type 16 (F)	5′-AAGGCCAACTAAATGTCAC-3′
(R)	5′-CTGCTTTTATACTAACCGG-3′
Type 18 (F)	5′-ACCTTAATGAAAAACCACGA-3′
(R)	5′-CGTCGTTGGAGTCGTTCCTG-3′
Type 33 (F)	5′-ATGATAGATGATGTAACGCC-3′
(R)	5′-GCACACTCCATGCGTATCAG-3′

were resolved on 2% agarose after ethidium bromide staining (Fig. 3A–E).

Amplimer details for type specific PCR and β-globin taken from published papers previously and were obtained through Microsynth.

Comparing the two techniques, RFLP with restriction enzyme Rsa I and PCR with type specific primers, we found that RFLP using Rsa-I can detect co-infections simultaneously and more consistently than individual typing with type specific primers and was cost-effective as it saves the total cost of second PCR for HPV typing with type specific primers (Aadrian et al., 1990). Use of nested PCR with degenerate primers have been described by various workers to be extremely sensitive means of detecting a wide range of HPV types (Broker et al., 2001; Harwood et al., 1999, Pizzighella et al., 1995). RFLP of the inner nested PCR product of MY09/11 primers using *Bst E11* and a double digest of *Pst I* and *Bgl I* (Patti et al., 2002) detected 10 high-risk anogenital HPV types. Both the methods were able to detect broad range of HPV types. Most of the PCR–RFLP studies show either use of multiple restriction enzymes with two rounds of amplification. Whereas, method described in this paper, was found to be precise, with only one round of amplification, followed by RFLP using a restriction enzyme, detected the five most prevalent HPV types commonly associated with cervical abnormalities and cancer. In conclusion, this method is less cumbersome and user-friendly for the

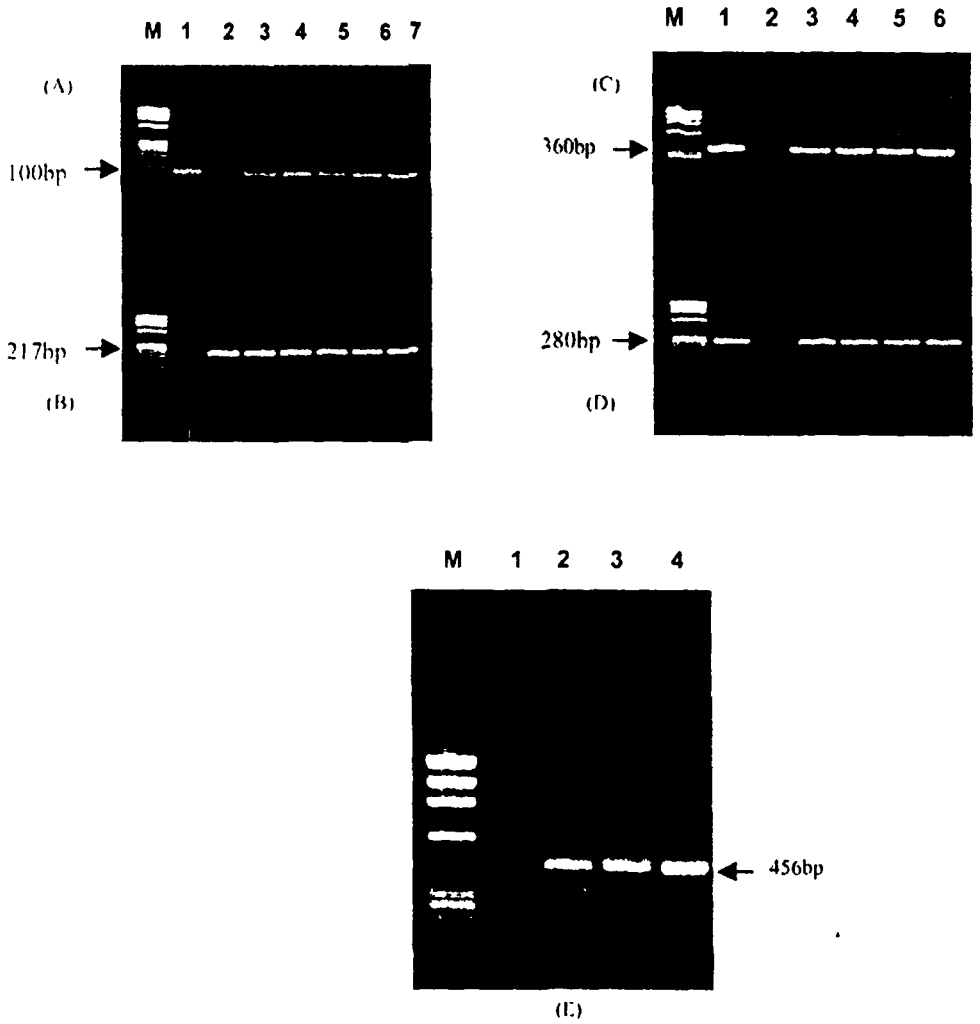


Fig. 3. Showing HPV types 18, 16, 11, 6, and 33 (A–E respectively) amplified using type specific primer, resolved on 2% agarose with ϕX 174 (Hae III digested) marker with positive controls (lanes 1 in A, C, and D and lane 2 in B and E) and negative controls (lanes 2 in A, C, and D, lane 1 in B and E).

detection of HPV DNA from cervical swabs, both at clinical and research level.

Acknowledgements

We express our thanks for excellent technical assistance to Mr. Mahendra Pratap, Technical Officer.

References

Aadrian, J C., Van den Brule, A.J C., Chris, J.L.M.M., Victor, B., Peter, K., Walboomers, J M M., 1990. Rapid detection of human papillomavirus in cervical scrapes by combined general primer-mediated and type specific polymerase chain reaction. J. Clin. Microbiol. 28 (12), 2739–2743.

Beaudenon, S., Kremsdorf, D., Croissant, O., Jablonska, S., Wain-Hobson, S., Orth, G., 1986. A novel type of human papillomavirus associated with genital neoplasias. Nature 321, 246–249.

Beaudenon, S., Praetorius, F., Fremsdorf, D., Lutzner, M., Worsaae, N., Pehau-Arnaudet, G., Orth, G., 1987. A new type of human papillomavirus associated with oral focal epithelial hyperplasia. J. Invest. Dermatol. 88, 132–135.

Bosch, F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen, A M., Peto, J., Schiffman, M.H., Moreno, V., Kurman, R., Shah, K.V., Alihonou, E., Bayo, S., Mockhtar, H.C., Chicreon, S., Daudt, A., Delosrios, E. Ghadirian, P., Kitinya, G.N., Koulibaly, M., Ngelangel, C., Tintore, L.M.P., Riosdalenz, J.L., Sarjadi, A., Schneider, A., Tafur, L., Teyssie, A.R., Rolan, P.A., Torroella, M., Tapia, A.V., Wabings, H R., Zatonski, W., Sylla, B., Vizcaino, P., Magnin, D., Kaldor, J., Greer, C., Wheeler, C., 1995. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. J. Nat. Cancer Inst. 87, 796–802.

Broker, T.R., Jin, G., Croom-Rivers, A., Bragg, S M., Richardson, M., Chow, L.T., Vermund, S.H., Alvarez, R D., Pappas, P G., Squires, K.E., Hoesley, C.J., 2001. Viral latency: the papillomavirus model. Dev. Biol. 106, 443–451.

Clifford, G.M., Smith, J.S., Plummer, M., Munoz, N., Franceschi, S., 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br. J. Cancer 88, 63–73.

Cole, S T., Danos, O., 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of

papillomaviruses and repeated structure of the E6 and E7 gene products J Mol Biol 193 599–608

Cole, S T Streeck, R L, 1986 Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer J Virol 58, 991–995

de Roda Husman A M, Walboomers, J M M, Van Den Brule, A J C, Meijer C J L M, Snijders, P J F, 1997 The use of general primers GP5 and GP6 elongated at their 3′ ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR J Gen Virol 76 1057–1062

Favre M Obalek, S, Jablonska, S, Orth, G, 1989 Human papillomavirus type 28 (HPV28), an HPV3 related type associated with skin warts J Virol 63, 4905

Gissmann L Diehl, V, Schulz-Loulon, H J, zur Hausen, H, 1982 Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma J Virol 44, 393–400

Gopalkrishna V, Francis, A, Sharma, J K., Das, B C, 1992 A simple and rapid method of high quantity DNA isolation from cervical scrapes for detection of human papilloamvirus infection. J Virol Methods 36 63–72

Gravitt, P E, Peyton, C L, Apple, R J, Wheeler, C M, 1998 Genotyping of 27 Human Papillomavirus types by using L1 consensus PCR products by a single hybridization reverse line blot detection method J Clin Microbiol 36, 3020–3027

Halbert, C L Demers, G W, Galloway, D A, 1992 The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells J Virol 66, 2125–2134

Harwood, C A, Spink, P J, Surentheron, T, Leigh, I M, De Villiers, E M, McGregor J M, Proby, C M, Breuer, J, 1999 Degenerate and nested PCR a highly sensitive method for detection of human papillomavirus infection in cutaneous warts J Clin Microbiol 37 (11), 3545–3555

Hildesheim. A, Schiffman, M H, Gravitt, P E, Glass, A G, Greer, C E, Zhang, T Scott, S R, Rush, B B, Lawler, P, Sherman, M E, Kurman, R J, Manos, M M, 1994 Persistence of type specific human papillomavirus infection among cytologically normal women J Infect Dis 169, 235–240

Jacobs, M V, Snijders, P J F, Voorhorst, F J, Dilner, J, Forslund, O, Johansson, B, von Knebel Doeberitz, M, Meijer, C J L M, Meyer, T, Nindi I, Pfister, H, Stockfleth, E, Strand, A, Wadell, G, Walboomers, J M M, 1999 Reliable high-risk HPV DNA testing by polymerase chain reaction an intramethod comparision J Clin Pathol 52, 498 503

Liaw K-L Hsing, A, Chen, C-J, Schiffman, M, Zhang, T, Hsieh, C Y Greer, C, You S-L, Huang, T, Wu, T, O'Leary, T, Seidman, J, Blot, W, Meinert, C, Manos, M, 1995 Human papillomavirus and cervical intraepithelial neoplasia a case control study in Taiwan Int J Cancer 62, 565–571

Manos, M M, Ting, Y, Wright, D K., Lewis, A J, Broker, T R, Wolinski, S M, 1989 Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses Cancer Cells 7, 209–214

Saiki, R K., Chang, C A, Levenson, C H, Warren, T C, Boehm, C D, Kazaian, H H 1988 Diagnosis of sickle cell anemia and β thalassemia with enzymatically amplified DNA and non-radioactive allele-specific oligonucleotide probes N Engl Med 319, 537–541

Nieminen, P, Aho, M, Vesterinen, E G, Vahen, A, Scares, V R X, Paovonen, J, 1991 Natural history of HPV infection preliminary results of a cohort study [abstract] in 1991 papillomavirus workshop Seattle, WA 77

Patti, K., Kathleen, M, Anna-Lise, W, 2002 The use of nested polymerase chain reaction and restriction fragment length polymorphism for the detection and typing of mucosal human papillomaviruses in samples containing low copy numbers of viral DNA J Virol Methods 159, 159–170

Pizzighella, S, Pisoni, G, Bevilacqua, F, Vaona, A, Palu, G, 1995 Simultaneous polymerase chain reaction detection and restriction typing for the diagnosis of human genital papillomavirus infection J Virol Methods 55, 245–256

Qu, W, Jiang, G, Cruz, Y, Chang, C J, Ho, G Y F, Klein, R, Burk, R D, 1997 PCR detection of Human papillomavirus comparision between MY09/MY11 and GP5+GP6+ primer systems J Clin Microbiol 35 (6), 1304–1310

Schwarz, E, Dürst, M, Demankowski, G, Lattermaun, O, Zech, E, Wolfsberger, E, Suhai, S, zur Hausen, H, 1983 The sequence and genome organization of genital human papillomavirus type 6b EMBO J 2, 2341–2348

Seedorf, K., Krämmer, G, Dürst, M, Suhai, S, Röwekamp, W, 1985 Human papillomavirus type 16 DNA sequence Virology 145, 181–185

Sillins, I, Wang, Z, Avall-Lundqvist, E, Frankendal, B, Vikmanis, U, Sapp, M, Schiller, J T, Dillner, J, 1999 Serological evidence for protection by HPV type 6 infection against HPV type 16 cervical carcinogenesis J Gen Virol 80, 2931–2936

Van den Brule, A J C, Class, H C J, du Maine, M, Mechlers, W J G, Helmerhorst, T, Quint, W G V, Lindeman, J, Meijer, C J L M 1989 Use of anticontamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies J Med Virol 29, 20–27